REMARKS

In the Office Action dated November 20, 2008, claims 38 and 42-47 are pending and under consideration. Claims 38, 42-43 and 47 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Richardson et al. (Environmental Mutagenesis 3: 545-553, 1981) ("Richardson") in view of Griffith et al. (U.S. Patent No. 5,354,996, referred to herein as "the '996 patent"). Claims 44-46 are rejected under 35 U.S.C. §103(a) as unpatentable over Richardson in view of the '996 patent and further in view of Nikiforov et al. (U.S. Patent No. 5,610,287, referred to herein as "the '287 patent").

Interview

A telephone interview with Examiner Cheu and Supervisory Examiner Mark Shibuya was conducted on December 2, 2008. Principal features and advantages of the present invention and distinctions over Richardson were discussed during the interview. Applicants, through the undersigned, wish to thank the Examiners for the courtesy and assistance extended to Applicants during the interview.

This Response is consistent with the discussion during the interview and addresses each of the Examiner's rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claim Amendments

Independent claims 38 and 47 have been amended to recite "ions of metals", to more clearly reflect that the claimed invention relates to the simultaneous detection of multiple metals in environmental samples. Support for this amendment is found in the specification, e.g., on page 23, line 1, and is further discussed below.

New claim 48 is added, drawn to a method of determining toxicity of an environmental sample associated with the presence of ions of metals. Support for this claim is found in the title of the application and throughout the specification, including the discussion of toxicants such as heavy metals on page 7, lines 18-31, for example.

No new matter is introduced by the foregoing amendments.

35 U.S.C. §103(a) – Obviousness

The Examiner has rejected Claims 38, 42 to 43 and 47 under 35 U.S.C. §103(a) as allegedly unpatentable over Richardson in view of the '996 patent.

The Examiner has alleged that one of skill in the art would have been motivated by the teachings of the '996 patent to use the method of Richardson to measure metal ions in environmental samples. In this regard, the '996 patent allegedly teaches the importance of monitoring levels of heavy metal ions in the environment, e.g. in aquatic samples.

Applicants respectfully submit that the instant method is distinguished from that of Richardson because Richardson's method does not, among other things, simultaneously detect multiple metals in an environmental sample; and further, Richardson does not teach how to simultaneously detect multiple metals in an environmental sample. As submitted previously, Richardson relates to the measurement of the concentration of metal ion, i.e., a single metal ion at a time, in a laboratory-contrived solution.

However, the Examiner seems to suggest in the Office Action, at for example, page 6, paragraph 2, that the claim language does not clearly specify that the instant method is directed to the detection of multiple metals in a sample, but can be construed as encompassing detecting multiple metal ions at different times, which allegedly reads on Richardson's method. It

appeared that the Examiner's position in this regard was confirmed during the telephone interview on December 2, 2008.

In order to advance prosecution, Applicants have amended the claims to recite "ions of metals". Support for the term "metals" can be found throughout the specification, e.g. at page 23, line 1. Additionally, the specification contains no teaching or suggestion that the instant method must be repeated for the detection of each individual metals in a sample. To the contrary, the instant method is a one-step method for simultaneously detecting the presence of different metals in one sample. The result obtained by the present method is achieved by the combined displacement of intercalated fluorescent dye by all of the metals present in the sample.

Applicants further respectfully submit that it is an inherent feature of environmental samples that they would contain multiple metals. This notion is supported by, for example, the quoted extracts from the following articles, copies of which are provided herewith as **Exhibits A-C**.

Ince et al. (1999) Arch. Environ. Contam. Toxicol. 36: 365-372, at page 365:

> "Aquatic organisms in natural water systems, however, are generally exposed to mixtures of metals, which may substantially multiply, suppress, or add the effects of single components."

Posthuma et al. (1997) Ecotoxicology and Environmental Safety 38: 108-121, at page 108:

> "Organisms inhabiting polluted habitants are almost always chronically exlposed to various toxicants simultaneously."

Rachlin and Grosso (1993) Arch. Environ. Contam. Toxicol. 24: 16-20, at page 16:

> "...examining the effects of divalent cations in various combinations is more representative, of the actual environmental problems faced by organisms, than are single metal studies. This recognition results from the realization that environmental loadings of cations from anthropogenic sources rarely involve single cation contributions, and if they do the introduced cation will interact with a host of chemicals

> native to the receiving system. Thus, organisms potentially impacted by these toxicants face a multiple rather than a single toxicant insult."

Accordingly, Applicants respectfully submit that upon reading the '996 patent, which itself teaches the presence of multiple metals in environmental samples (see column 1, lines 42 to 43), one of skill in the art would not have been motivated to employ the method of Richardson, which measures a single metal in a laboratory sample, to simultaneously detect multiple metals in an environmental sample.

Furthermore, Applicants reassert that those skilled in the art would not have expected the method of Richardson to provide a meaningful measure of toxicity of an environmental sample due to the co-existence of multiple metals in the sample. In this connection, Applicants direct the Examiner's attention to the previous submission, including the Declaration of Professor Cristobal Guillermo dos Remedios, in support of Applicants' position in this regard.

In particular, Applicants submit that multiple metals, which may be present in an environmental sample, do not necessarily merely have an additive toxic effect. The presence of one metal often has either a synergistic or an antagonistic effect on the activity of another metal in the sample, and *vice-versa*. This phenomenon affects the overall toxicity of the sample. For example, if Metal X and Metal Y each alone cause 1 arbitrary unit of toxicity, the additive effect would result in about 2 units, a synergistic effect would result in >2 units, and an antagonistic effect would result in <2 units. This was explained in the Declaration, Paragraph 9.

To facilitate the Examiner's understanding of interactions between metals, Applicants direct the Examiner's attention to the quoted passages from the following documents (copies also enclosed as **Exhibits D-F and Exhibit C**), which teach or hypothesize why synergy and/or antagonism occurs upon contacting whole organisms or cells with combinations of heavy metals.

Moulder (1980) Marine Biology 59: 193-200, at page 195:

"It would appear that under these conditions copper is in some way protecting the gammarids from mercury poisoning. There are 3 possible reasons for this: (i) copper may occupy binding sites on the surface of the gammarid which would otherwise be occupied by mercury, thus reducing uptake and accumulation; (ii) a copper-mercury complex might be formed in sea water reducing availability to the gammarid; (iii) copper may in some way detoxify mercury within the tissue."

And at page 199:

"Both mercury and copper ions are known to bind to sulphydryl (SH) groups [...]. The toxicity date (Table 1, Fig. 1) indicates the enormous tolerance that *Gammarus duebeni* shows to copper compared with that to inorganic mercury. It is proposed that the grammarid may be able to "use" this tolerance to copper by selectively blocking the binding sites on the surface of the gammarid with copper in preference to mercury when the 2 metals are presented together in solution."

Vrnaken et al. (1988) Marine Environmental Research 26: 161-179, at page 177:

"At present we only can state, as is generally accepted (Babich & Stotzky, 1983), that antagonistic interactions result from competition between the chemicals for sites on the cell surfaces, whereas synergism is indicative for an increased permeability of the plasma membrane."

Braék and Jensen (1976) J. Exp. Mar. Biol. Ecol. 25: 37-50, at page 49:

"The antagonism may be caused by a competition of the two metals for a common uptake site."

Rachlin and Grosso (1993) Arch. Environ. Contam. Toxicol. 24: 16-20, at page 20:

"That metals compete for adsorption sites on the plasma membrane and evoke a toxicity response by affecting what traverses the membrane is well known..."

And also at page 20:

"The non-linear response to these cation combinations, when examined at each 24 h time interval (Table 3, Figure 2), indicates that the toxic response is not uniform over time. This is constituent with a response resulting from alterations in membrane permaeability. That cations interact with sulfhydryl groups on proteinaceous membranes to produce –S-metal-S- bridges, which can then alter membrane permeability was demonstrated by Rothstein (1959), and Simkiss (1979), and Simkiss (1979) indicated the importance of proteins and lipoproteins in trapping

metals, which provides a mechanism for removing cations from the cell as a detoxification mechanism."

Applicants further respectfully submit that the prevailing view in the art around the time of the conception of the present invention was that synergistic or antagonistic effects of multiple metal toxicants could *only* be measured with live whole organism or cell-based assay systems. Molecular assay systems, e.g. "naked" nucleic acid based systems, were thought *not* to be useful for this purpose due to the lack of *inter alia*, cellular machinery (e.g. enzymes), cell/organelle membranes and associated adsorption sites, intracellular and trans-membrane import/export mechanisms and intracellular and membrane-bound receptor molecules. Up until the conception of the present invention and its reduction to practice, a molecular assay system would have been expected by the person skilled in the art to only be capable of measuring an additive effect. Notably, the publications in the field, prior to the filing of the present application, only documented live whole organism or cell-based assays, where the toxicity of an environmental sample is determined by assessing the effect of sample exposure on the viability and/or phenotype of the organism/cell. See Paragraphs 7-8 and 10 of the Declaration.

The instant inventors, however, have unexpectedly shown that a naked nucleic acid based assay is able to detect synergy and antagonism in mixtures of metals (see Paragraph 10 of the Declaration), which had been thought by those skilled in the art to being capable of measured by only live whole organism or cell-based assays.

Accordingly, Applicants respectfully submit not only the cited prior art references do not teach or suggest the claimed invention, the claimed invention also provides a rapid and accurate means for measuring the toxicity of an environmental sample containing multiple heavy metal toxicants – a result that is unexpected to those skilled in the art.

To more clearly delineate the features and advantages of the claimed methods, Applicants have added claim 48, directed to a method of determining toxicity of an environmental sample associated with the presence of ions of metals.

In view of the foregoing, it is respectfully submitted that the methods, as presently claimed, are not obvious over the combination of Richardson and the '996 patent. Withdrawal of the rejection under 35 U.S.C. §103(a) based on these references is respectfully requested.

With regard to the obviousness rejection of Claims 44 to 46 based on Richardson in view of the '996 patent and further in view of the '287 patent, Applicants respectfully submit that the fundamental deficiencies of Richardson and the '996 patent are not cured by the '287 patent. Therefore, withdrawal of the rejection under 35 U.S.C. §103(a) based on the combination of these three references is respectfully requested.

Conclusion

In view of the foregoing amendments and remarks, it is firmly believed that the present application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

Xiaochun Zhu, Ph.D.
Registration No. 56,311

Scully, Scott, Murphy & Presser, P.C.
400 Garden City Plaza-Suite 300
Garden City, New York 11530
(516) 742-4343
XZ:ab

Enc. Exhibits A-F

EXHIBIT A

Arch. Environ. Contam. Toxicol. 36, 365–372 (1999)

ARCHIVES OF
Environmental
Contamination
and Toxicology

Assessment of Toxic Interactions of Heavy Metals in Binary Mixtures: A Statistical Approach

N. H. Ince,[1] N. Dirilgen,[2] I. G. Apikyan,[1] G. Tezcanli,[1] B. Üstün[1]

[1] Institute of Environmental Sciences, Boğaziçi University, 80815 Bebek-Istanbul, Turkey
[2] Department of Chemistry, Boğaziçi University, 80815 Bebek-Istanbul, Turkey

Received: 5 February 1998/Accepted: 28 November 1998

Abstract. Toxicity of zinc, copper, cobalt, and chromium ions and their binary interactions were studied at varying test levels by using a battery of two tests, Microtox and duckweed with *Vibrio fisheri* and *Lemna minor* as test organisms, respectively. The type of toxic interaction at each test combination was assessed by a statistical approach based on testing the null hypothesis of "additive toxicity" at 95% confidence level. The interactions were called "antagonistic," "additive," or "synergistic" in accordance with the statistical significance and the sign of the difference between the tested hypothesis and the value of the observed toxicity at the binary test level concerned. In the majority of the combinations studied by the two bioassays, the interactions were of antagonistic nature. Additive toxicity was the next frequently predicted interaction in both test results, the frequency being much higher in Microtox responses than in those of duckweed. Finally, synergism was found to be a rare interaction in Microtox results, but totally unlikely in duckweed within the selected test combinations.

Owing to the remarkable advances in biomonitoring technologies during the last decade, toxicity testing using rapid, reliable, and inexpensive methods has been promoted as a desirable alternative to chemical-specific tools in assessing and controlling the discharge of toxic chemicals into the environment. As a consequence, numerous such tests have been proposed and shown as potential alternatives to the traditional bioassays (*e.g.*, fish and invertebrates), which are relatively complicated and time-consuming, requiring as well trained personnel, well-equipped laboratories, and high costs of operation. On the other hand, both the traditional and the currently developed short-term toxicity testing methods (*e.g.*, microbioassays and higher plant tests) have shown that there is no single bioassay or organism that is responsive at all times to all toxicants or mixtures of toxicants, discharged or released into aquatic systems. This realization has led to the "battery" concept of using at least two bioassays (with species of different origin) to ascertain the ecological impacts of chemical releases and to develop an early warning and remedial strategy for toxicity assessment in aquatic environments (Munawar *et al.* 1989; Ince 1996).

Metals have received particular attention among other chemicals in toxicity studies, owing to their uniquely adverse effects on aquatic life forms as a result of their natural and anthropogenic release into such ecosystems. The majority of published data concerning toxicity testing of heavy metals is focused on single metal effects, comparison of indicator sensitivities, and correlation of results from various bioassays to those from the conventional fish and daphnia tests (McFetters *et al.* 1983; Coleman and Qureshi 1985; Reteuna *et al.* 1989; Wang 1991). Aquatic organisms in natural water systems, however, are generally exposed to mixtures of metals, which may substantially multiply, suppress, or add the effects of single components. Several methods have been proposed during the last 10–15 years to predict the toxicity of complex mixtures from single-species laboratory test results. Among these are the "application factor method," the "distribution function method," and "the structure activity relationships-QUASAR method" (OECD 1992, 1995; Aldenberg and Slob 1993; EU 1995; Pedersen and Petersen 1996). However, many of these methods, especially the QSARs, are largely applicable to organic pollutants or classes of organic compounds. Similar relationships for inorganic species are rare in the literature, although toxic interactions of heavy metals in aquatic systems have recently been the focus of many ecotoxicologists. Generally, the basis of most prediction models describing such effects is "additive toxicity," which assumes that toxicity of a mixture is nearly equivalent to the sum of the single-species toxicity of its components (Hermens and Leeuwagh 1982; Abel 1989; Ribo and Rogers 1990; Pedersen and Petersen 1996). Published data in the literature are highly agreeable with the additivity principle, though deviations toward repressed or lowered effects, *i.e.*, "antagonism," are reported as well (Sellers and Ram 1985; Pedersen and Petersen 1996). Deviations toward magnification of additive effects, *i.e.*, "synergism," is rare in the literature.

The purpose of this study was to assess the interactive toxicity of zinc (II), copper (II), cobalt (II), and chromium (VI) in various binary mixtures using the observed toxicity of single metal species and that of their binary combinations. All data were generated in a battery of two simple and well-defined

Correspondence to: N. H. Ince

bioassays: Microtox® and duckweed. The selection of the battery was insignificant within the scope of the study, although factors such as simplicity, cost-effectiveness, reproducibility, and availability of test organisms in the laboratory were important. Moreover, the wide applicability of both *V. fisheri* and *L. minor* as indicators in various ecotoxicological studies has justified our selection. Because the luminescence of *V. fisheri* is directly associated with respiratory activity, it is reported to provide a good indicator of metabolic actions and therefore of the general cytotoxicity of a compound or a mixture of compounds (McConkey *et al.* 1997). Furthermore, the common duckweed *L. minor* is widely used as a surrogate of all vuscular and higher plant species due to its significant advantages in toxicity testing. Among these are preliminary evaluation of contaminant impacts on primary productivity, ability to tolerate turbid samples, unstable environmental conditions, and high sensitivity to heavy metal toxicity, which is favorably compared to that of fish (Lewis 1995; Greenberg *et al.* 1993; Wang 1986).

The method of assessing the interactive toxicity of binary metal combinations in this study involved the following scheme: (1) testing of single metal samples in increasing concentrations, followed by reducing the dose-response data (bioluminescence in Microtox and growth in duckweed) to EC_{50}s; (2) testing of various binary combinations prepared by keeping the concentration of one metal constant while varying the other along a range where responses were easily detectable and reproducible; and (3) assessing the interactive metal effects as "additive," "antagonistic," or "synergistic" by statistical testing of the difference between observed and calculated responses. Calculations were based on the addition or multiplication respectively, of the toxicity units (Microtox) or relative growth rates (duckweed) of single metal ions, detected at the ion concentration corresponding to that used in preparing the mixture.

Materials and Methods

Reagents and Supplies

Microtox reagents and supplies as specified in the standard basic protocol (Microbics 1992) were obtained from Microbics Corp. (Carlsbad, CA). Duckweed plants were subcultured from original stocks, maintained in Cekmece Nuclear Research Center Laboratories, Istanbul. Illumination of stock and test cultures was made with fluorescent tubes of Philips TLD 36W/54. All chemicals used in sample preparation were analytical grade.

Samples

Stock solutions of 1,000 mg L^{-1} of $CoCl_2$ and $K_2Cr_2O_7$ were prepared from reagent grades in deionized water. The dichromate solution was acidified with sulfuric acid (to pH = 3.5) to maintain a relatively stable Cr (VI) species (Skoog *et al.* 1997; Villaescusa *et al.* 1997). High-purity chloride and nitrate salts of zinc and copper were made from pure metals by dissolving them respectively in 1:1 (v:v) HCl (12 M) and 1:1 (v:v) HNO_3 (16 M), followed by dilution to 1,000 mg L^{-1} in deionized water.

Microtox Assay

The Microtox testing system is based on photometric determination of the reduction in the light output of freeze-dried luminescent bacteria at 0, 5, and 15 min of exposure to a toxicant or mixture of toxicants. A Model-500 Analyzer, equipped with an array of sample wells for holding dilutions of bacterial suspensions at constant temperature (15°C) and a photometer for measuring the light output of *V. fisheri*, was used in the study. Single metal test levels were selected by initial screening of five serial dilutions (dilution factor = 5) of each stock solution to determine the test range, where the effective concentration should be sought. Toxicity was tested at increasing concentrations of the metal ion, starting from the lowest detectable, or the least toxic sample. Binary mixtures of each pair were prepared at a minimum of two combinations, using a selection from single metal test levels. The basic protocol was followed for sample preparation, osmotic adjustment, and serial dilution procedures (Microbics 1992). Toxicity was measured as percent inhibition of light emission (corrected for the light loss in the control) before and after 15-min incubation of the reconstituted bacteria in four serial dilutions of the single and binary metal test concentrations. (5-min light readings were disregarded due to their lack of reproducibility.)

Data Analyses. Estimations of EC_{50}, the effective toxicant concentration corresponding to 50% inhibition were carried out as percentage of initial metal concentration by using data-reduction software provided by the manufacturing company, and based on the Gamma function, which is equal to the ratio of light loss to light remaining. For the binary solutions, EC_{50}s were generated similarly, but by setting the initial toxicant concentration to the sum of the two metal ions in the mixture.

Duckweed Assay

Duckweed is a widespread, fast-growing, and free-floating aquatic plant, and the temperature zone species *L. minor* is most popular in toxicity testing due to its high sensitivity and simplicity of cultivation (Lewis 1995). Several different test protocols are available for duckweed as reported by Wang (1991), Taraldsen and Norberg-King (1990), Cowgill and Milazzo (1989), and Huebert and Shay (1993). The method used in this study was based on static conditions. Duckweed plants were subcultured from an original stock (maintained since 1987) in full-strength Jacob culture medium (McLay 1976). Bright green and healthy duckweeds were selected from the subcultured stock for use in toxicity testing. Sixty milligrams (WW) of a sample from the selected subculture of *L. minor* was measured out and rinsed carefully with distilled water. Duckweeds were then placed in 200 ml of metal test solution contained in a 300-ml jar and covered with aluminum foil to exclude side lighting and with watch glasses to prevent evaporation. The test solutions were adjusted to pH = 6.0 with 0.1 M KOH or HCl. The stock and test cultures were continuously illuminated with preheat daylight fluorescent tubes having an intensity of 40 $\mu E\ m^{-2}s^{-1}$ at plant level. Temperature was kept at 25–27°C. On the seventh day of frond incubation, plants were washed three times with distilled water and weighed. The experimental set for each testing scheme consisted of control samples and five replicates of the test sample. Single metal test levels were set by sequential increase of an initial value of 0.2 mg L^{-1} of each metal ion (except chromium) until statistically sufficient data were available to estimate the value of EC_{50} by regression analyses. Binary test levels were selected with respect to the estimated EC_{50} of each metal component. The test parameter growth was measured as biomass at the end of 7 days of incubation. Toxicity was recorded as percent inhibition of growth (relative to control) in *L. minor* as a result of 7-day exposure to the toxicant in its growth medium.

Data Analyses. Growth was defined in terms of relative growth rate (RGR) based on the formula: $RGR = (\ln W_f - \ln W_i)/t$, where W_i and

Table 1. Fifteen-minute Microtox responses to single metal test concentrations[a]

Metal Ion	Test Concentration (mg L^{-1})	15-min EC_{50} (%)	Toxicity Units ($100/EC_{50}$)	15-min EC_{50} (mg L^{-1})
Zinc (II)	2	79.50	1.26 (0.09–2.58)	**1.62** (1.01–4.53)
	4	40.02	2.50 (1.62–2.90)	[1.5[b]; 1.20[c]; 1.6[d]]
	5	32.40	3.09 (2.34–3.70)	
	6	27.00	3.70 (2.43–7.40)	
	7	23.14	4.32 (3.73–5.21)	
	8	20.23	4.94 (3.69–7.56)	
Copper (II)	0.5	54.00	1.85 (1.78–1.91)	**0.30** (0.24–0.34)
	1	27.00	3.70 (3.66–3.74)	[0.70[b]; 0.31[c]; 0.6[e]]
	1.5	17.34	5.77 (5.26–6.29)	
	2	20.23	7.15 (6.61–7.32)	
Cobalt (II)	60	55.40	1.81 (1.28–2.53)	**32.71** (25.75–46.30)
	80	39.86	2.51 (2.06–3.00)	
	90	36.68	2.73 (2.39–3.14)	
Chromium (VI)	60	60.83	1.64 (1.38–1.94)	**35.20** (27.90–48.30)
	70	46.58	2.15 (1.75–2.64)	[23[b]; 32.7[c]; 47.6[e]; 23.7[f]; 52[g]]
	80	45.63	2.19 (1.41–3.10)	

[a] Numbers within parenthesis are 95% confidence intervals of reported means. Figures in bold character in the last column refers to an average value of EC_{50} estimated by use of Equation 1. Values in brackets are from the works of others
[b] McGrath 1988
[c] Microbics 1992
[d] Hinwood *et al.* 1987
[e] Codina *et al.* 1998
[f] Villaescusa *et al.* 1997 (pH = 4.6)
[g] Villaescusa *et al.* 1997 (pH = 5.1)

W_f are the fresh weights at time 0 and t, respectively (Ericsson *et al.* 1982). The EC_{50}, or the effective toxicant concentration to induce 50% growth inhibition was estimated by regression analyses of RGR (as percentage of control sets) and the applied metal ion concentration in mg L^{-1}, by using a statistical package program (SYSTAT 1992).

Statistical Modeling

The interaction of test metals in each test mixture was assessed by comparing the observed toxicity at the i^{th} test level and at the concentration $(x + y)_i$ (where x and y are the concentrations of the first and second metal ions, respectively) with the value of the null hypothesis at that level, defined as "the sum of the toxicity indices of the two metals, tested previously at x and y." For the Microtox data, the null hypothesis was evaluated by the addition of toxicity units, defined as $TU = 100/EC_{50}$, where EC_{50} is expressed as percentage of initial sample concentration. For the duckweed data, evaluation of the null hypothesis was based on multiplication of RGRs of each metal as percentage of control, using Colby's formula as reviewed by Rai *et al.* (1981). Hence, toxic interactions at each binary test level was assessed by statistical testing of the two null hypotheses, TU_H and RGR_H, defined by Equation 1 and Equation 2 for Microtox and duckweed data, respectively:

$$H_0 \text{ (Microtox): } TU_H(x + y)_i = TU_{x,i} + TU_{y,i} \qquad \text{(Eq. 1)}$$

$$H_0 \text{ (duckweed): } RGR_H(x + y)_i = \frac{(RGR_x)_i(RGR_y)_i}{100} \qquad \text{(Eq. 2)}$$

where $(x + y)_i$ is the i^{th} test combination in the mixture in mass concentration units, $TU_{x,i}$, $TU_{y,i}$ are the Microtox toxicity units of the metal ions, observed at singular test concentrations x_i and y_i, and $(RGR_x)_i$, $(RGR_y)_i$ are the duckweed relative growth (as %) of each metal ion, recorded at the x_i^{th} and y_i^{th} singular concentrations.

Statistical testing of the null hypotheses involved (1) computation of the difference (TU_{DIFF} for Microtox and RGR_{DIFF} for duckweed) between null hypothesis and the observed response at each test level by $TU_{DIFF} = TU_H - TU_{obs}$, and $RGR_{DIFF} = RGR_{obs} - RGR_H$; (2) estimation of a student *t* for each difference using the standard errors of estimate (SE) of added and observed results, respectively; and (3) comparison of the estimated *t* with the tabulated *t* value (t_{sign}) at the total degrees of freedom arising from subtracting observed means of TU and RGR from calculated means to determine if the difference is statistically significant at 95% confidence level.

Calculation of the standard error of TU_{DIFF}, RGR_{DIFF}, and *t* for each test level was based on basic statistical concepts (Havilcek and Crain 1988; Miller and Miller 1993), and the use of Equations 3 and 4, respectively:

$$SE_{DIFF,i} = \sqrt{(SE_{obs})_i^2 + (SE_H)_i^2} \qquad \text{(Eq. 3)}$$

$$t = \frac{TU_{DIFF},\ RGR_{DIFF}}{SE_{DIFF}} \qquad \text{(Eq 4)}$$

where $SE_{DIFF,i}$ is the standard error of estimate of TU_{DIFF} or RGR_{DIFF} for the i^{th} test level, and $(SE_{obs})_i$, $(SE_H)_i$ are the standard errors of estimate of observed and calculated (hypothesis) toxicity units or relative growth rates of the mixture at i, respectively.

Results and Discussion

Single Metal Toxicity

Microtox Test Results: Table 1 lists the test levels of the Microtox assay and the 15-min responses of *V. fisheri* at each level, reported as the effective concentration, EC_{50} (as percentage of sample strength), and 100 times its reciprocal, *i.e.*,

toxicity units (TU). The conversion of EC_{50} to TU is due to our preference of TU as the toxicity parameter in our statistical modeling, for its convenience in arithmetic operations as a direct indicator of the relative toxicity of a test sample. All results reflect the mean of at least two replicates.

From the estimated values of EC_{50} at each test level, an effective concentration for the metal ion was calculated in terms of mass units, by using Equation 5, where C_i and $(\%EC_{50})_i$ are the i^{th} test concentration (mg L^{-1}) and the corresponding EC_{50} as percentage of C_i, respectively; and n is the number of test samples.

$$EC_{50}\ (\text{mg L}^{-1}) = \sum_i \frac{C_i(\%EC_{50} \times 10^{-2})_i}{n} \qquad \text{(Eq. 5)}$$

The toxic trend of the test metals were found to increase with increasing sample concentration and in the order Cu > Zn > Co > Cr, which is in agreement with the data of McGrath (1988), who reported the toxicity of Cu, Zn, and Cr to Microtox test species.

A selection of data from the literature is presented in the last column of Table 1 for comparative purposes. The variations in the listed values of EC_{50}s are typical of most toxicological studies, arising from vulnerability of testing sublethal responses in living organisms and variations in experimental conditions. It is further important that in the Microtox assay, alterations in the standard methodology with respect to pH and osmotic adjustment medium, differences in batch of bacteria, the initial toxicant concentration, and the method of data reduction are potential sources of diversities between different laboratories (Hinwood *et al.* 1987). Good agreement was found for copper and zinc between our results and most of the reported work of others, while our data for the EC_{50} of chromium is slightly higher than those found in the literature. Some researchers have suggested the use of sucrose as an alternative to NaCl for osmotic adjustment to prevent the formation of metal complexes with chloride ions (Hinwood *et al.* 1987). Others recommended that pH of the test medium be varied in accordance with the chemistry involved, and the exposure time be expanded to observe delayed responses, claiming that *V. fisheri* responds "slowly" to heavy metals (Villaescusa *et al.* 1997). We believe that the relatively low sensitivity of the Microtox reagents to Cr in this study arises from a combination of all these factors. It must be particularly associated with the fact that the acidic stock solution of $K_2Cr_2O_7$ was readjusted to pH = 6, when it was impossible to determine a test range with the original stock during preliminary screening. Upon this pH change, the distribution of Cr (VI) species in solution is expected to be shifted from the dominant $HCrO_4^-$ to CrO_4^{-2}, a situation which is reported to increase the 15-min Microtox EC_{50} of Cr (VI) by one order of magnitude (Villaescusa *et al.* 1997). However, since binary mixtures were tested at exactly the same conditions as those of single metals, we believed that this shift would not effect the prediction of the interactive toxicity of chromium mixtures by the proposed model. Note that no data were available in the literature for the EC_{50} of Co (II) to compare with ours.

Duckweed Results: Toxicity of single test metals to *L. minor* are reported in Table 2, both as percent of relative growth (of control) RGR, and as EC_{50} (mg L^{-1}), estimated by regression analysis of the dose-response data.

Table 2. Seven-day responses of *L. minor* to single metal test concentrations

Metal Ion	Test Concentration (mg L^{-1})	Observed RGR (%)	Toxicity (EC_{50} mg L^{-1})
Zinc (II)	0.2	93 ± 6.8	**9.6**
	0.5	89 ± 5.3	
	1	85 ± 7.3	
	2	81 ± 4.4	
	5	67 ± 4.5	
	10	53 ± 3.9	
Copper (II)	0.2	87 ± 3.6	**1.5**
	0.5	80 ± 2.9	
	1	70 ± 3.6	
	2	31 ± 2.3	
Cobalt (II)	0.2	100 ± 4.5	**15.7**
	0.5	86 ± 4.1	
	1	86 ± 5.9	
	2	79 ± 8.2	
	5	71 ± 4.9	
	10	61 ± 3.4	
	15	53 ± 3.6	
	20	46 ± 6.4	
Chromium (VI)	1	86 ± 5.3	**8.5**
	2	104 ± 7.6	
	5	81 ± 5.8	
	10	32 ± 6.4	

The growth of *L. minor* in all experiments was inhibited with increasing concentrations of the test metal. Morphological symptoms (yellowing and disintegration of fronds) of metal toxicity were evident at high concentrations of Cr and Cu ions, namely 10 and 2 mg L^{-1}, respectively, while an apparent stimulation of growth at low concentrations of Co (0.2 mg L^{-1}) was observed. The toxicity of the test metals were found to increase in the order Cu > Cr = Zn > Co. No literature data were available for comparison with ours, except for those of Wang (1991) who reported, however, 96-h EC_{50}s of the heavy metals he studied.

Comparison of Single Metal Responses: From Tables 1 and 2, one can conclude that copper was the most toxic metal to both species, while the degree of sensitivity to the other three metals was diverse. The relative toxicity of the test metals to each test species is presented in Figure 1 as EC_{50}.

It was found that *L. minor* was twice as sensitive to Co and four times more sensitive to Cr than *V. fisheri,* while it was about eight and five times less sensitive to Zn and Cu, respectively. The pH adjusted Cr (VI) stock solution in the Microtox test (as discussed earlier) must be largely responsible for the reported difference in Cr toxicity. The larger toxicity of Co to *L. minor* might be due to the larger effect of cobalt complexes with chloride (than chloride complexes with the other metals) in the Microtox test medium, and metal chloride complexes are reported to reduce the toxicity of metal ions to *V. fisheri* (Hinwood *et al.* 1987). On the other hand, Cu and Zn were much more toxic to Microtox reagents than to duckweed, in spite of the presence of chloride ions. This can be attributed to the different nature of the organisms, related with metal-specific uptake mechanisms.

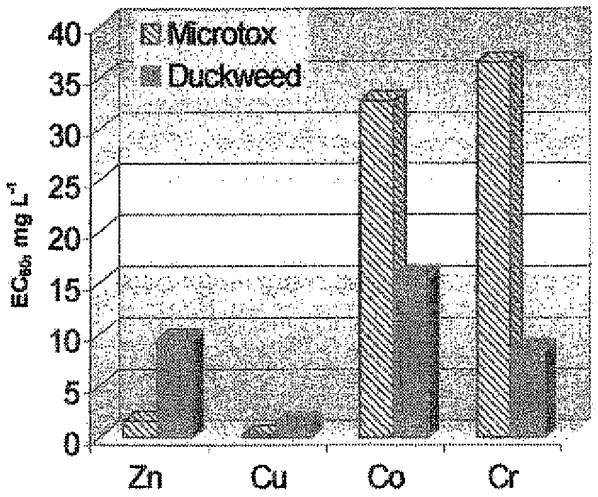

Fig. 1. Comparison of the relative toxicity of test metals to *V. fisheri* and *L. minor*

Binary Metal Interactions

The type of binary interaction at each test level was assessed by using Equation 3 and Equation 4, and with respect to the statistical significance of TU_{DIFF} and RGR_{DIFF}, in such a way that:

- If the difference was positive and statistically significant (*i.e.*, $t > t_{sign}$), the interaction was called "antagonistic," implying that the toxicity of the mixture is lower than additive toxicity.
- If the difference was negative and statistically significant, the interaction was called "synergistic," implying that the toxicity of the mixture is higher than additive toxicity.
- If the difference was statistically insignificant (irrespective of its sign), the interaction was called "additive."

Microtox Test Results: The value of binary toxicity at each test combination is reported as toxicity units, TU_{obs} ($100/EC_{50}$) in Table 3 (the second column). Calculated toxicity indices (TU_H) for each test level, differences between calculated and observed results (TU_{DIFF}), and the predicted binary interactions are presented in the last three columns of Table 3, respectively. Out of six binary mixtures with 34 test combinations, the distribution of "antagonistic" and "additive" interactions were close (41% and 38%, respectively), while the incidence of "synergistic" interactions was less than one third of each (11%). It was surprising that Cu, with the highest degree of toxicity, interacted antagonistically with Zn and Cr at most of the test levels, except at 0.5 + 6 and 1 + 6 levels as Cu + Zn (where the interactions were additive), and at 0.5 + 60 level as Cu + Cr (where the interaction was synergistic). The interaction between Co and Cr can also be classified as antagonistic, although an additive response was predicted at one of the three test levels. On the other hand, it was found that additivity is the best description for the interaction of Zn with both of Cr and Co, since it was consistently predicted at all test levels. Finally, synergism was descriptive of Cu-Co interactions only, being predicted at most of the test levels, except at 1.5 + 60 as Cu + Co, where the metals were found to interact additively.

Table 3. Observed and calculated microtox responses at binary test combinations x:y, and single metal concentrations x, y, respectively (predicted interaction types)

Metal Pair at (x:y)		Observed Toxicity, TU_{Obs} (df = 4)[c]	Calculated Toxicity, TU_H (df = 8)[c]	Difference, TU_{DIFF}[a] (df = 12)[c]		Interactive Effect[b]
x ($mg\ L^{-1}$)	y ($mg\ L^{-1}$)	($100/EC_{50}$)	$TU_x + TU_y$	$TU_H - TU_{Obs}$		
Zn	Cu					
4	1	4.13 ± 0.56	6.20 ± 1.01	2.07	S	ANT
4	1.5	4.37 ± 0.59	8.27 ± 1.11	3.90	S	ANT
4	2	5.61 ± 0.68	9.65 ± 1.00	4.04	S	ANT
5	0.5	3.50 ± 0.30	4.94 ± 0.74	1.44	S	ANT
5	1	4.00 ± 0.25	6.79 ± 0.72	2.79	S	ANT
5	1.5	5.08 ± 0.42	8.86 ± 1.19	3.78	S	ANT
6	0.5	4.19 ± 0.60	5.55 ± 2.55	1.36	I	ADD
6	1	5.47 ± 0.51	7.40 ± 2.52	1.93	I	ADD
7	1	5.52 ± 0.31	8.02 ± 0.78	2.77	S	ANT
7	1.5	7.14 ± 0.35	10.09 ± 1.25	2.95	S	ANT
7	2	8.18 ± 0.52	11.47 ± 1.09	3.29	S	ANT
Zn	Co					
2	60	2.79 ± 1.69	3.05 ± 1.27	0.26	I	ADD
2	80	3.10 ± 0.56	3.75 ± 1.11	0.65	I	ADD
2	90	3.76 ± 0.79	3.97 ± 1.01	0.21	I	ADD
4	80	5.50 ± 1.04	5.01 ± 1.17	−0.49	I	ADD
5	90	6.23 ± 1.96	5.82 ± 2.86	−0.41	I	ADD
Zn	Cr					
2	60	2.55 ± 0.26	2.90 ± 1.53	0.35	I	ADD
2	70	2.91 ± 0.08	3.41 ± 1.69	0.50	I	ADD
4	60	4.62 ± 0.10	4.14 ± 0.92	−0.51	I	ADD
4	70	5.36 ± 0.34	4.65 ± 2.86	−0.71	I	ADD
Cr	Cu					
60	0.5	4.04 ± 0.32	3.49 ± 0.35	−0.55	S	SYN
60	1	4.45 ± 0.29	5.34 ± 0.32	0.89	S	ANT
60	1.5	4.63 ± 0.22	7.41 ± 0.79	2.78	S	ANT
70	1.5	4.79 ± 0.40	7.92 ± 0.96	3.13	S	ANT
Cr	Co					
60	60	2.70 ± 0.17	3.45 ± 0.90	0.75	S	ANT
70	60	2.41 ± 0.15	3.96 ± 1.07	1.55	S	ANT
70	80	4.62 ± 0.40	4.15 ± 0.91	0.47	I	ADD
Co	Cu					
60	0.5	4.66 ± 0.57	3.66 ± 0.69	−1.00	S	SYN
60	1	6.63 ± 0.51	5.51 ± 0.66	−1.12	S	SYN
60	1.5	7.81 ± 1.68	7.58 ± 1.14	−0.23	I	ADD
80	0.5	5.50 ± 0.73	4.36 ± 0.53	−1.14	S	SYN
80	1	7.40 ± 0.82	6.21 ± 0.51	−1.19	S	SYN
90	0.5	7.25 ± 2.39	4.53 ± 0.41	−2.72	S	SYN
90	1	8.34 ± 0.94	6.43 ± 0.42	−1.91	S	SYN

[a] S = statistically significant, I = statistically insignificant
[b] ANT = antagonistic, ADD = additive, SYN = synergistic
[c] df = degrees of freedom

Duckweed Test Results: The binary test combinations and the observed toxicity, RGR (as % of control) at each level are reported in third and fourth columns of Table 4. The toxic interactions of metal pairs at the combinations $(x:y)_i$ were evaluated by calculating the value of "additive toxicity" $(RGR_H)_i$ of the concerned mixture, using the individual RGRs of its metal components at x and y.

Calculated toxicity indices, their differences from observed results, and the predicted interactions are reported in the last

Table 4. Observed and calculated toxicity as RGR to *L. minor* at binary combinations x:y, and single metal test levels x, y, respectively (predicted interaction types)

Metal Pair at (x:y)		Observed Toxicity, RGR_{obs} (df = 4)[c]	Calculated Toxicity, RGR_H (RGR_x*RGR_y)/100 (df = 5–8)[c]	Difference, RGR_{DIFF}[a] RGR_{obs} − RGR_H (df = 9–11)[c]	Interactive Effect[b]
x (mg L^{-1})	y (mg L^{-1})				
Zn	Cu				
0.5	0.5	99 ± 5	71 ± 4	28 S	ANT
1	1	86 ± 4	59 ± 6	27 S	ANT
5	1	95 ± 6	47 ± 4	54 S	ANT
10	1	88 ± 4	37 ± 3	51 S	ANT
2	2	62 ± 5	25 ± 2	37 S	ANT
10	2	59 ± 8	17 ± 2	42 S	ANT
20	2	47 ± 7	NC[d]	—	—
Zn	Co				
0.2	0.2	88 ± 4	93 ± 8	−5 I	ADD
0.5	0.5	74 ± .5	77 ± 6	−3 I	ADD
1	1	68 ± 7	73 ± 8	−5 I	ADD
1	10	78 ± 3	52 ± 5	26 S	ANT
1	20	83 ± 4	39 ± 6	44 S	ANT
2	2	59 ± 5	64 ± 8	−5 I	ADD
5	5	87 ± 6	48 ± 5	39 S	ANT
5	10	85 ± 6	41 ± 4	44 S	ANT
5	20	98 ± 8	31 ± 5	67 S	ANT
10	10	73 ± 9	32 ± 3	41 S	ANT
10	20	83 ± 8	24 ± 4	59 S	ANT
10	40	76 ± 7	NC	—	—
Zn	Cr				
1	1	121 ± 5	73 ± 6	48 S	ANT
1	2	114 ± 11	88 ± 8	26 S	ANT
1	5	96 ± 5	69 ± 5	27 S	ANT
1	20	12 ± 1	NC	—	—
2	1	102 ± 7	70 ± 6	32 S	ANT
2	2	92 ± 8	84 ± 5	8 I	ADD
2	10	59 ± 1	26 ± 0.3	33 S	ANT
5	1	80 ± 2	58 ± 3	22 S	ANT
5	5	72 ± 3	54 ± 5	18 S	ANT
5	10	30 ± 2	21 ± 3	9 S	ANT
10	10	24 ± 2	17 ± 2	7 S	ANT
15	15	7 ± 0.5	NC	—	—
Cu	Cr				
1	1	104 ± 8	60 ± 5	44 S	ANT
1	10	83 ± 7	22 ± 5	61 S	ANT
1	20	2 ± 0.3	NC	—	—
2	2	98 ± 9	32 ± 5	66 S	ANT
2	10	46 ± 4	9.9 ± 2	36 S	ANT
2	20	5 ± 1	NC	—	—
5	5	49 ± 3	NC	—	—
10	10	18 ± 4	NC	—	—
Cr	Co				
1	1	122 ± 10	47 ± 4	75 S	ANT
2	2	118 ± 8	82 ± 4	36 S	ANT
5	1	116 ± 11	70 ± 6	46 S	ANT
5	5	89 ± 8	57 ± 4	32 S	ANT
5	10	97 ± 9	49 ± 5	48 S	ANT
10	1	65 ± 6	28 ± 3	37 S	ANT
10	10	44 ± 4	19 ± 3	25 S	ANT
15	15	19 ± 2	NC	—	—
20	10	17 ± 1	NC	—	—
Co	Cu				
0.5	0.5	83 ± 3	69 ± 4	14 S	ANT
1	1	73 ± 6	60 ± 5	13 I	ADD
1	10	96 ± 9	43 ± 3	53 S	ANT
1	20	96 ± 10	32 ± 5	64 S	ANT
2	2	61 ± 3	24 ± 3	37 S	ANT
2	10	78 ± 6	19 ± 1	54 S	ANT
2	20	78 ± 7	14 ± 2	64 S	ANT

[a] S = statistically significant, I = statistically insignificant
[b] ANT = antagonistic, ADD = additive, SYN = synergistic
[c] df = degrees of freedom, ranging from 5 to 8 for RGR_H and 9 to 11 for RGR_{DIFF}
[d] NC = not calculated

three columns of Table 4. Note that although a response was observed for all binary test levels, RGR_H is not reported for some of them. This is because either of the metal ion concentrations in the mixture was much higher than the EC_{50} of the corresponding single metal ion, to which the response of *L. minor* at that concentration level was death. In such cases, therefore, where RGR_i of one of the binary metal ions was missing (referred to as NC in Table 4), the value of the null hypothesis (additive toxicity) could not be evaluated (by Equation 2), and the interactive effects were not determined (*e.g.*, for mixtures where Cr > 10 mg L^{-1}, and Cu > 2 mg L^{-1}, etc.).

The data and reported interaction types in Table 4 show that no synergistic interactions were found as a result of the assessment procedure described above. Aside from the unpredictable 10 cases (referred as NC), out of 45 assessed interactions, 39 were found to be antagonistic, and six were additive.

The predicted antagonistic interactions at all levels of Zn + Cu combinations suggest that Zn suppress the toxic effects of Cu, as was formerly reported (Dirilgen and Inel 1994a). Moreover, yellowing and disintegration of fronds were observed at 20 + 2 mg L^{-1} of Zn + Cu mixture. The combinations of Cu + Co (except at 1 + 1 mg L^{-1}), Cu + Cr, Zn + Cr, and Co + Cr (except at 2 + 2 mg L^{-1}) were also found to result in growth responses that are larger than predicted by Equation 4, exhibiting antagonism, as well. For combinations of Cu + Cr at equal concentrations of each metal from 1 to 5 mg L^{-1}, Cr was not only found to suppress the inhibitory effect of Cu but even to stimulate the growth of *L. minor*, which could not, however, survive in the test medium where Cu and Cr were combined as 1 + 20 and 2 + 20 mg L^{-1}.

Zn and Co being least toxic to *L. minor* and eliciting toxic effects only at high concentrations, as reported by Dirilgen and Inel (1994b), were found to interact antagonistically at most test levels except at combinations of low concentrations of both (0.2–2 mg L^{-1}), at which they interacted additively. On the other hand, low concentrations of Cr combined with either Co or Zn were found to stimulate the growth of *L. minor*, implying that the growth-inhibiting effect of Cr can be effectively suppressed by a proper fraction of Co or Zn in the test medium. Nevertheless, the suppression by Zn was found to diminish when Cr existed at higher concentrations; *e.g.*, at 10 + 10 mg L^{-1} and 15 + 15 mg L^{-1} as Cr + Zn, the type of interaction however remaining antagonistic for all test levels.

Assessment of the Predicted Interactions in the Battery: The interpretation of the data as a whole reveals that the interaction of Cu with Zn and Cr is antagonistic, while that of Zn with Co and Cr is antagonistic-additive. Based on the disagreement of the predictions for Co-Cu pair (synergism by Microtox and antagonism by duckweed), it is concluded that the toxic effect of a mixture of these two metals is highly dependent on the nature and type of the test organism. The synergistic effect on *V. fisheri* can be explained by the fact that as a bacterium, almost all cellular functions are directly exposed to the toxicant once cellular membranes are breached (McConkey *et al.* 1997). In contrast, *L. minor* is expected to be protected by the plant-typical structural compartmentalization, the cell wall being an additional barrier to the toxicant uptake (McConkey *et al.* 1997). This important structural difference between the two test species also explains the larger number of antagonistic and the absence of synergistic interactions in the duckweed data, as opposed to fewer antagonistic and some synergistic predictions in the Microtox.

Conclusions

A battery of two bioassays (Microtox and duckweed) was used to generate the required data for predicting the interactive effects of heavy metals in binary mixtures by a novel method based on statistical testing of additive toxicity as a null hypothesis. It was found that 87% of the predictions made with the duckweed data was antagonistic and 13% was additive, while Microtox data provided 41% antagonistic, 38% additive, and 11% synergistic interactions. The total fraction of antagonistic responses in the battery was 66%, implying that suppression of toxic effects is highly probable when heavy metals are combined in binary mixtures. This is a contradiction to the commonly predicted additive toxicity reported in the literature. On the other hand, through a comparative evaluation of the results within the battery, it can be concluded that bacterial species are more vulnerable to heavy metal toxicity than plant species even in the presence of binary mixtures, due to the structural differences that effect the toxicant uptake mechanisms.

We feel that the true merit of the proposed model lies in the fact that it is unbiased for additive toxicity and can be used safely to predict the interactive toxicity of more complex mixtures, once the toxic components are identified.

Acknowledgments. The research was funded by Norwest Soil Laboratories, Edmonton, Canada, and Boğaziçi University Research Fund, Istanbul, Turkey, through grant no. 96Y0014. All assistance and support are highly appreciated.

References

Abel PD (1989) Water pollution biology. Ellis Horwood, West Sussex, England

Aidenberg T, Slob W (1993) Confidence limits for hazardous concentrations based on logistically distributed NOEC toxicity data. Ecotoxicol Environ Safety 25:48–63

Codina JC, Munoz MA, Cazorla FM, Perez-Garcia A, Morinigo MA, DeVicente A (1998) The inhibition of methanogenic activity from anaerobic domestic sludges as a simple toxicity bioassay. Wat Res 32(4):1338–1342

Coleman RN, Qureshi AA (1985) Microtox and *Spirillum volutants* tests for assessing toxicity of environmental samples. Bull Environ Contam Toxicol 26:150–156

Cowgill UM, Milazzo DP (1989) The culturing and testing of two species of duckweed. In: Cowgill UM, Williams LR (eds) Aquatic toxicity and hazard assessment. ASTM, STP 1027 12:379–391

Dirilgen N, Inel Y (1994a) Effects of zinc and copper on growth and metal accumulation in duckweed, *Lemna minor*. Bull Environ Contam Toxicol 53:442–449

Dirilgen N, Inel Y (1994b) Cobalt-copper and cobalt-zinc effects on duckweed growth and metal accumulation. J Environ Sci Health 29A(1):63–81

Ericsson T, Larsson CM, Tillberg E (1982) Growth responses of *Lemna* to different levels of nitrogen limitation. Z Pflanzenphysiol 105:331–340

EU (1995) Environmental risk assessment of new and existing substances. Technical guidance document, draft, May

Greenberg BM, Huang XD, Dixon DG, Ren L, McConkey BJ, Duxbury CL (1993) Quantitative structure activity relationships for the photoinduced toxicity of polycyclic aromatic hydrocarbons to duckweed—a preliminary model. In: Gorsuch JW, Dwyer EJ, Ingesoll CG, La Point TW (eds) Environmental Toxicology and Risk Assessment. ASTM, STP 1216, 2:369–378

Havilcek LL, Crain RD (1988) Practical statistics for the physical sciences. American Chemical Society, Washington, DC

Hermens J, Leeuwagh P (1982) Joint toxicity of mixtures of 8 and 24 chemicals to the guppy (*Poecilia reticulata*). Ecotox Environ Safety 6:302

Hinwood AL, McCormick MJ, McCormick RJ (1987) Evaluation of the Microtox technique for assessment of toxicity in waters and wastewaters. Report prepared for Environment Protection Authority of Victoria, Australia

Huebert DB, Shay JM (1993) Considerations in the assessment of toxicity using duckweeds. Environ Toxicol Chem 12:481–483

Ince NH (1996) An overview of microbioassay techniques. In: Ince N (ed) Proceedings of First International Workshop on Alternative Bioassay Techniques Suitable for Toxicity Monitoring in the Black Sea, April 24–26 1995, Istanbul. Boğaziçi University, Istanbul, p 171

Lewis MA (1995) Use of freshwater plants for phytotoxicity testing: a review. Environ Pollut 87:319–336

McConkey BJ, Duxbury CL, Dixon DG, Greenberg BM (1997) Toxicity of a PAH photooxidation product to the bacteria *Photobacterium phosphoreum* and the duckweed *Lemna gibba:* effects of phenathrene and its primary photoproduct phenanthrene quinone. Environ Toxicol Chem 16(5):892–899

McFetters GA, Bond PJ, Olson SB, Tchan YT (1983) A comparison of microbial bioassays for the detection of aquatic toxicants. Wat Res 17(12):1757–1762

McGrath MJ (1988) Assessment of the Microtox toxicity analyzer as a screening tool for activated sludge wastewater treatment plant effluents. MS thesis, University of Massachusetts, Amherst

McLay CL (1976) The effect of pH on the population growth of three duckweeds: *Spirodela oligorrhiza, Lemna minor* and *Wolffia arrhiza.* Freshwat Biol 6:125–136

Microbics Co. (1992) Microtox manual. Carlsbad, CA

Miller JC, Miller JN (1993) Statistics for analytical chemistry. Ellis Horwood Limited and Prentice Hall, Chichester

Munawar M, Dixon G, Mayfield CI, Reynoldson T, Sadar MH (1989) Preface. In: Munawar M, Dixon G, Mayfield CI, Reynoldson T, Sadar MH (eds) Environmental bioassay techniques and their application. Kluwer Academic Publishers, Dordecht

OECD (1992) Report of the OECD workshop on the extrapolation of laboratory aquatic toxicity data to the real environment. OECD Environment Monographs 59, OECD, Paris

OECD (1995) Guidance document for aquatic effects assessment. OECD Environment Monographs 92, OECD, Paris

Pedersen F, Petersen GI (1996) Variability of species sensitivity to complex mixtures. Wat Sci Tech 33(6):109–119

Rai LC, Gaur JP, Kumar HD (1981) Pycology and heavy metal pollution. Biol Rev 56:99–151

Reteuna C, Vaseur P, Cabridenc R (1989) Performances of three bacterial assays in toxicity assessment. Hydrobiologia 188/189:149–153

Ribo JM, Rogers F (1990) Toxicity of mixtures of aquatic contaminants using luminescent bacteria bioassay. Toxicity Assess 5:135–152

Sellers KE, Ram NM (1985) Toxicity assessment of binary metal mixtures using biolumunious bacteria. Microbics Reference Library, no. 55

Skoog DA, West DM, Holler FJ (1997) Analytical chemistry: an introduction, 6th ed. Saunders Golden Sunburst Series, Saunders College Publications, Philadelphia

SYSTAT, (1987) The system for statistics. Evanston, IL

Taraldsen J, Norberg-King TJ (1990) New method for determining effluent toxicity using duckweed (*Lemna minor*). Environ Toxicol Chem 9:761–767

Villaescusa I, Marti S, Matas C, Martinez M, Ribo JM (1997) Chromium (VI) toxicity to luminescent bacteria. Environ Toxicol Chem 16(5):871–874

Wang W (1986) Toxicity tests of aquatic pollutants by using common duckweed. Environ Pollut Series B 11:1–14

Wang W (1991) Literature review on higher plants for toxicity testing. Water, Air Soil Pol 59:381–400

EXHIBIT B

ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 38, 108–121 (1997)
ARTICLE NO. ES971568

Single and Joint Toxic Effects of Copper and Zinc on Reproduction of *Enchytraeus crypticus* in Relation to Sorption of Metals in Soils

L. Posthuma, R. Baerselman, R. P. M. Van Veen, and E. M. Dirven-Van Breemen

Laboratory for Ecotoxicology (RIVM/ECO), National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Received February 28, 1997

Joint toxic effects of copper and zinc were studied in the terrestrial worm *Enchytraeus crypticus* (Westheide and Graefe) (Oligochaeta, Annelida). Animals were exposed in OECD artificial soil. Sublethal toxicity was judged by effects on reproduction. Metals were applied singly or in binary mixtures. Observed effects were compared with effects expected from simple similar action (concentration addition), by recalculation of metal concentrations in toxic units. Exposure of the worms was quantified with body concentrations and with external concentrations (total, extractable, soluble). The observed joint effect was similar to concentration additive when judged by external concentrations and less than concentration additive for body concentrations. This difference is attributable to interactions among metals during sorption to soil and during uptake. Copper reduced the sorption of zinc to soil, but copper sorption was inert for zinc addition. Zinc uptake from the soil solution was stimulated by copper, but copper uptake was not stimulated by zinc. Joint effects of toxicants to soil biota are partly determined by interactions outside the organism, as a result of dissimilarity between total and bioavailable concentrations. The design of joint toxicity studies in terrestrial systems is discussed with special reference to metal sorption in soils, experimental methodology, and laboratory practice. The joint toxic effect of copper and zinc for *E. crypticus* was of similar magnitude as found in studies with aquatic species exposed to metal mixtures. © 1997 Academic Press

INTRODUCTION

Organisms inhabiting polluted habitats are almost always chronically exposed to various toxicants simultaneously. Ecotoxicological risk assessment and setting quality standards for soils are, however, based on laboratory toxicity data with single-substance exposure (*e.g.,* Van Straalen and Denneman, 1989; Wagner and Løkke, 1991; Van Straalen and Løkke, 1997). This introduces various uncertainties, which are usually accounted for by the introduction of safety factors. One of the uncertainties bears on the joint effects of chemicals. Joint effects may be similar to or stronger or weaker than expected from effects of separate exposure. They may be inevitable; for example, if a raw material is composed of various chemicals as holds for metal ores or oil or they may be specific, such as the possibly synergistic interaction between some pesticides and estrogen receptors (Arnold *et al.,* 1996). A firm scientific basis is needed to cope with joint toxic effects in risk assessment procedures.

Compared to the aquatic compartment (*e.g.,* Enserink *et al.,* 1991) joint toxicity data are particularly scarce for soil biota. Joint-effect studies with soil organisms are mostly restricted to analyses of effects at only few exposure concentrations. In such cases, the data are insufficient to apply appropriate toxicological joint effect models. Moreover, the existing studies mostly neglect that in soil the overall joint effect is the result of interactions at various levels, inside and outside the body. Sorption to soil may render a fraction of the chemical inaccessible for uptake by organisms, sorption being influenced by both soil characteristics and the presence of other chemicals. For risk assessment purposes, it is crucial to acknowledge all relevant interaction levels, in particular, since extrapolation of joint toxicity effects between soil types may depend on fixed interaction sequences among chemicals in soil.

Joint effect studies require specification of an "expected" joint effect, usually on the basis of data on the effects of chemicals applied singly, with which observed joint effects are compared. For ecotoxicity studies in soil, the formulation of this "expectation" not only requires data on interactions in the toxicological sense, but also on the influence of sorption. In the present study the following basic assumptions and models were used to quantify expected effects (1–3) and sorption and exposure (4):

1. Expected joint effects were calculated on the basis of simple similar action as null model [same mode of action (hereafter MoA), but no interaction; see, *e.g.,* Hewlett and Plackett, 1959];

2. The (relative) toxic unit concept was applied (see, *e.g.,* Sprague, 1970; De March, 1987);

3. No detailed investigations were made into the MoA of the metals;

4. Exposure was quantified in different ways to assess the contribution of each interaction level separately, *viz.* by expressing exposure using body concentrations, and using total, extractable, and soluble soil concentrations. Using these expressions of exposure, assessment was made of whether the joint effect was influenced by (a) interactions among the

0147-6513/97 $25.00

chemicals outside the organism, (b) interactions during uptake (toxicokinetics), or (c) interactions inside the body at the target site(s) (toxicodynamics) (Calamari and Alabaster, 1980). Moreover, interactions in soil were assessed directly by comparison of sorption isotherms.

The hypotheses, models, assumptions, and study design are worked out under Materials and Methods.

The present study focused on joint toxicity of a binary metal mixture of copper and zinc for an oligochaete worm species, *Enchytraeus crypticus,* in an artificial soil substrate. The artificial soil substrate was chosen to rule out soil quality variation within the study, as this affects metal sorption and interactions outside the body. Data are presented on joint effects and on interactions in soil and during uptake. Juvenile production was used as toxicity endpoint. Exposure duration was 4 weeks, which is a compromise between chronic, life-span exposure [life span is 73–90 days under the prevailing conditions (Westheide and Graefe, 1992)] and practical feasibility. In addition, methodological aspects related to the study design and the use of the artificial soil substrate and implications for risk assessment are briefly discussed.

MATERIAL AND METHODS

Enchytraeids

The culture of *E. crypticus* (Westheide and Graefe) was kindly provided by Dr. J. Römbke from a culture at Osnabrück. The culture has been maintained in the laboratory for many generations. Cultures are kept at 17°C on a soil extract agar, in the dark. The substrate was made by shaking 1 kg dried peaty soil in 3 L demineralized water (overnight), followed by paper filtration and water addition until a volume of 2 L was reached. Agar (30 g Oxoid No. 3) was dissolved in this soil extract. This mixture was heated in a pressure cooker at 120°C for 20 min and poured into petri dishes. These were sterilized at 200°C before use. Every 4 weeks four fresh plates were inoculated with 25 adults each to maintain the culture. Cultures were fed *ad libitum* with mixed oat meal, yeast, cod liver oil, and milk powder (3:1:1:1 on weight basis). For the experiment 3000 animals of similar age were needed. To this end, the number of inoculated dishes was extended, and adults were removed after 1 week.

Artificial Soil

Animals were exposed in artificial soil (modified from OECD 1984) (hereafter artisoil). The artisoil was made of 10% 0.5 mm ground *Sphagnum* peat, 20% kaoline clay, and 70% fine quartz sand (50% particles between 0.05 and 0.2 mm), proportionally mixed on a dry weight basis. The pH was set to approximately 6.0 by addition of fine powdered $CaCO_3$ (6.2 g/kg dry soil) to avoid the reproduction effects that occur at the low indigenous pH of the mixture (Dirven-Van Breemen *et al.,* 1994). Water (35%, w/w) was added to the dry mixture to obtain optimal humidity for the enchytraeids (Dirven-Van Breemen *et al.,* 1994). Metals were added as stock solution during this step (see below). To avoid differences in metal sorption between batches of artisoil, care was taken to obtain all constituents from homogeneous lots of base material, and the artisoil was made in a single mixing run. Prepared soil (with or without metals) was stored for 1 week, to equilibrate metal sorption and acidity.

Chemicals

Metals were added as aqueous stock solutions of chloride salts (copper, $CuCl_2 \cdot H_2O$, Merck, purity > 99%, MW 170.5 g/mol; zinc, $ZnCl_2$, Merck, purity >98%, MW 136.28 g/mol). To balance the anion concentrations among treatments (see below), sodium chloride was added in treatment-specific amounts as aqueous stock (NaCl, Merck, p.a., MW = 58.44 g/mol).

Joint Effect Model

Toxicologists have defined four classes of joint effects based on the similarity or dissimilarity of the MoA's and on the presence or absence of (chemical) interactions (see, *e.g.,* Hewlett and Plackett, 1959). For each class, the expected joint effect is characterized by a specific "reference point" or null model, to which observed effects are compared. In ecotoxicological research the justification for choosing any null model is often weak, since various sites of interaction—both inside and outside the body—may determine the joint effect and since commonly information on MoA's is absent. For the present investigation, effect observations were compared to one of the possible null models, *viz.* "Simple Similar Action" (hereafter SSA, *sensu* Hewlett and Plackett, 1959). The reference point of SSA was calculated after scaling the concentrations of the chemicals. The common scale was defined by the relative toxicities of the chemicals (*e.g.,* the 4-week EC50 for reproductive activity). For each chemical, this concentration was defined as 1 toxic unit (TU, dimensionless), and other concentrations were expressed accordingly. Under SSA, the effect of x% in a mixture after n weeks of exposure is expected at a summed concentration of 1 TU. Observed joint effects were categorized either as similar to concentration additive ($TU_{observed} = 1$), or as more or less than concentration additive (resp. $TU_{observed} < 1$ or > 1).

Interaction Levels

It was hypothesized that joint effects could differ as a function of the number and relative influence of various interaction levels. Three interaction levels were addressed, *viz.* (1) interactions in soil, (2) interactions during uptake, and (3) interactions in the body. The hypothesis was tested first by comparing joint effects when calculated using total soil concentrations with those using body concentrations. In the former case the observed response is a result of the simultaneous action of all three interaction levels, while in the latter case the observed response is determined by interactions inside the body only. A difference between both suggests that interactions in soil, dur-

ing uptake, or both, have influenced the overall joint effect. In a similar way, the relevance of interactions during uptake was assessed, by comparing joint effects calculated using extractable or soluble concentrations with those using body concentrations. In this case, interactions in soils are already accounted for by the differences in extractability or solubility. Extractable and soluble concentrations were assumed to be available for uptake. Interactions in soil were determined also in a direct way, by comparison of extractability and solubility in the presence and absence of the second metal.

Experiment Design

First, range-finding experiments were performed in which the metals were added separately. The mixture experiment that followed consisted of three simultaneous treatment series, *viz.* with (A) copper singly, (B) zinc singly, and (C) equitoxic mixtures of copper and zinc (Table 1). A mixture is equitoxic if all chemicals are present at an equal fraction of their own EC50. Equitoxicity for series (C) was aimed at maximizing the sensitivity of the experiment for deviations of concentration additivity. Metal concentrations increased in geometric series, so as to cause reproduction effects between 0 and 100%, with an expected effect of 50% at concentration level 7 (A and B) or 6 (C). Six replicates were prepared for each treatment, four for observations on reproduction, and two for body concentration measurements.

Anion Balance

The use of metal chloride salts causes chloride concentrations to be different among treatments. This was compensated for by addition of equalizing amounts of aqueous NaCl stock. To judge the effect of salt addition, reproduction was compared between chloride-balanced controls and additional controls without any salt addition. Furthermore, a separate range-finding experiment was performed to study the effect of chlorides. To this end, a group of animals was exposed to different sodium chloride concentrations, under similar experimental conditions.

TABLE 1

Measured Metal Concentrations (Total, mg/kg dry wt) in Artisoil Used to Expose *Enchytraeus crypticus* to (A) Cu or (B) Zn Applied Singly or to (C) Equitoxic Mixtures of Cu and Zn

	Single (A and B)		Equitoxic mixutres (C)	
Level	Cu	Zn	Cu	Zn
1—Control	5	12	5	12
2	16	36	197	32
3	54	56	229	42
4	95	79	273	46
5	195	125	291	52
6	384	191	427	81
7	825	244	459	89
8	934	383	656	144
9	1333	499	832	173
10			894	182

Note. In addition to the chloride-balanced series A–C, reproduction was also studied in four non-chloride-balanced control replicates.

Exposure Conditions and Observations

Adult enchytraeids were randomly distributed in groups of 15 individuals and put in plastic containers (15 ml) filled with 10 g soil to start exposure. During exposure, the containers were kept at 17 ± 1°C in the dark. Animals were fed weekly with oat meal.

After exposure, the content of each container was diluted with water in a 100-ml vial. Approximately 10 ml of fixation dye (36% formaline solution mixed with Bengal red dye, 1:50 v/v) was added to allow for juvenile counts. Adult survival and juvenile numbers were counted. Juvenile numbers were expressed as juveniles per adult per week. For containers in which some mortality occurred, the assumption was made that all 15 adults had contributed to reproduction during the whole exposure period. The average reproduction in the chloride-balanced control replicates was set to 100%, and the reproduction per container was expressed as percentage of this.

Metal Concentrations

Metal concentrations in soils were determined in three ways after 1 week of equilibration. First, soil samples were digested in hot acid (HCl:HNO_3:deionized water 12:4:10 v/v/v, microwave heating) and filtered for determination of the total metal concentrations. Second, samples were shaken in deionized water (1:10 w/v) and filtered for determination of the water soluble concentrations. Third, the latter procedure was followed using 0.01 *M* $CaCl_2$ solution for determination of the exchangeable concentrations. The latter two methods were assumed to provide approximations of the metal fraction that can readily be taken up by worms (see Spurgeon and Hopkin, 1996; Posthuma and Notenboom, 1996). Soil metal concentrations were measured in digests or extracts using flame AAS and were expressed in milligrams per kilogram of dry weight or milligrams per liter for toxicity and sorption calculations, respectively.

Animals used for metal analyses in the body were captured by hand sorting. They voided their gut contents overnight on humid filter paper and were frozen, lyophilized, weighed, and digested following micromethods adapted from Van Straalen and Van Wensem (1986). Metal concentrations in the digests were determined with flame AAS for zinc and graphite furnace AAS for copper and were expressed in milligrams per kilogram of dry weight.

Statistics

Concentration–effect curves were assumed to be sigmoid and were described with the model of Haanstra *et al.* (1985)

$$y = C/(1 + e^{-b(\ln x - m)}), \quad (1)$$

where y is performance in soil with concentration x (%), C is the value for y under control conditions (%), b is the slope of the response curve at the EC50 concentration on a logit-log scale, and m is ln(EC50) (mg/kg or TU).

The model was fitted to the observations using the nonlinear curve-fitting program of GraphPad Prism (2.0) to obtain the parameter values.

The observed effects were compared to the effects expected under SSA for the four exposure expressions as explained above, *viz.* for total, soluble, and extractable soil concentrations and for body concentrations. To this end, the model was fitted to the reproduction data of the metals applied singly, to define TUs (separately for each exposure measure). Metal concentrations in the mixture series were expressed on the basis of these TUs, and the model was fitted to the data of the mixture series. The $TU_{observed}$ was compared to the $TU_{expected}$ under SSA.

After standardizing all concentrations to TUs, the shapes of the concentration–effect curves for the chemicals applied singly and in the mixture are similar if SSA truly applies. Shapes may differ, however, if chemicals have different MoA's or different sorption characteristics, as may apply to metal mixtures. Shape divergence may imply that the degree of dissimilarity between $TU_{observed}$ and $TU_{expected}$ depends on the exposure level. Therefore, additional calculations were made, *viz.* by studying (dis)similarity of slopes and by determining joint effects at other concentration levels than at the EC50 level only.

To determine (dis)similarity of slopes among curves, the data of those curves were normalized on the parameters C and m of Eq. (1). C was normalized by expressing juvenile numbers as percentages of the average control reproduction (set to 100%); m was normalized by expressing concentrations as fraction of the EC50. F tests were used to assess (dis)similarity of slopes between copper and zinc added separately and between copper or zinc and the mixture (Sokal and Rohlf, 1981). Significance of the F value between curves implies that shapes were different, since slope parameter b was the only free parameter. For cases where slope differences were indicated, joint effects were also judged at the level of the EC25 and EC10 to analyze how concentration dependency worked out quantitatively on $TU_{observed}$.

To determine whether observed joint effects differed significantly from the expected value of 1 TU (or $m = 0$), the data for both metals applied singly were normalized on 1 TU, whereas for the mixture series m was a free parameter. An F test was performed to compare curves. When slopes are not significantly different, significance of such F values implies that the EC50 ($= e^m$) of the mixture curve differs from 1 TU, since with standardized control reproduction and with similarity of slopes, the only free parameter is the EC50.

Sorption of metals to the soil was described by fitting the generalized Freundlich isotherm to the data on total and soluble or extractable concentrations.

$$C_S = K_F C_W^{\left(\frac{1}{n}\right)}, \quad (2)$$

where C_S is the total concentration in soil (mg/kg dry wt), K_F is the Freundlich adsorption constant (L/kg), C_W is the concentration in water or 0.01 M $CaCl_2$ extract (mg/L), and n is the shape parameter.

A high value for K_F indicates strong sorption to the solid phase, a high value for n indicates a high solid phase saturation rate at increasing soil concentrations. Parameter estimates were obtained by linear regression on log-transformed soil and liquid concentrations. To analyze metal uptake in the body, linear regressions on log-transformed soil and body concentrations were made. The slopes of the regression curves for sorption and body concentrations resulting from separate and mixed exposure were compared following Zar (1984), to assess the presence of interactions outside the body and during uptake.

RESULTS

Range Findings for Copper and Zinc

The 4-week EC50s for juvenile production for zinc and copper were 188 mg Zn/kg and 873 mg Cu/kg dry wt soil. The 95% confidence interval (CI) for zinc was 149–237. For copper the interval was undefined, due to the few observations indicating partial reproduction effects.

Exposure Conditions of Mixture Experiment

Actual rather than nominal metal concentrations were used to express exposure concentrations. Total metal concentrations in the artisoil are summarized in Table 1. Clean artisoil appeared to contain background concentrations of 12 mg zinc and 5 mg copper/kg dry wt.

The pH (1 M KCl) of the artisoil, measured at the start of the exposure, was 6.6 in control soil. In the treatments with the highest metal concentrations, initial pH values were 5.5 for the copper series, 6.4 for the zinc series, and 5.9 for the mixture series. After exposure, pH values reduced with 0.1–0.4 units.

The humidity, measured at the start of exposure, was near field capacity (33.9%).

Effects Related to Anion Balance

In the range-finding experiment with sodium chloride no reproduction effects were observed up to a concentration of approximately 1380 mg Cl^-/kg dry wt soil; the EC50 for reproduction was 2270 mg Cl^-/kg dry wt soil. Average juvenile production in (chloride-balanced) control conditions of the mixture experiment was 14 (SD 3) juveniles per worm per week (j/w/w), versus 23 j/w/w in controls without sodium chloride balance. This difference was significant (one-way ANOVA, $F = 5.3$, $P = 0.002$). These results suggest that the sodium chloride balance may have caused adverse reproduction effects in the mixture experiment, although the chloride

balance concentration of the mixture experiment (1420 mg Cl^-/kg) only slightly exceeded the NOEC.

Survival and Reproduction

Adult recovery varied between 60 and 100%, except for a single container in which no adults survived. The latter was skipped from the further analyses. The average number of adults recovered was 14.8 (SD 1.5). Recovery of less or more than 15 adults was attributed to mortality and incidental maturation of juveniles, respectively. It cannot be assured whether mortality or early maturation affected juvenile production in a replicate. Therefore, the calculations of juvenile per worm per week were based on the assumption that all adults contributed to juvenile production for 4 weeks; calculations (not provided) with alternate assumptions indicated that the findings on EC50s and joint effects were robust.

Separate and Joint Toxicity

Juvenile production reduced with increasing exposure concentration in the three treatment series. Table 2 summarizes the estimated parameters of the sigmoid response model for the four expressions of exposure.

As an example, Fig. 1 shows the three concentration–effect curves for total soil concentrations. Based on total soil concentrations it appeared that zinc was more toxic than copper (EC50s: 477 mg Cu/kg dry wt and 336 mg Zn/kg dry wt, F test, $P < 0.05$). This holds both when expressed as milligrams per kilogram or as millimoles per kilogram, since both metals have similar molecular weight. Both the juvenile production and the estimated EC50s were variable, which implies that TUs based on these curves are relative rather than absolute values. The EC50 of the mixture at the level of total concentrations was 1.2 TU (95% CI 1.00–1.46). The observed effect, resulting from interactions both inside and outside the body, did not differ significantly from the expected value under concentration addition (F value 2.05, df [3, 22], ns). There was no evidence for a concentration-dependent joint effect, since the slopes exhibited no significant differences, neither among copper and zinc nor between the separate metals and the mixture.

TABLE 2
Parameters of the Logistic Concentration–Effect Curves and Estimated EC Levels (with 95% confidence intervals, CI) for Different Exposure Expressions

		Model parameters					
Measurement	Exposure	C	b	m	ECx	95% CI	Deviation from CA
					EC50		
Total	Cu	109.9	−2.525[a]	6.167	477[a]	(345–658) mg/kg	
	Zn	94.78	−4.371[a]	5.818	336[b]	(266–425) mg/kg	
	Mixture	95.44	−4.835[a]	0.192	1.212	(1.003–1.463) TU	$P > 0.1$
					EC50		
Soluble	Cu	102.6	−4.496[a]	1.933	6.91[a]	(4.25–11.23) mg/kg	
	Zn	92.94	−5.391[a]	1.362	3.90[a]	(3.08–4.94) mg/kg	
	Mixture	92.81	−2.132[a]	0.089	1.09	(0.48–2.48) TU	$P > 0.25$
					EC50		
Extractable	Cu	117.3	−0.898[a]	0.610	1.84[a]	(0.42–8.04) mg/kg	
	Zn	95.12	−2.871[a]	3.010	20.29[b]	(14.35–28.67) mg/kg	
	Mixture	96.53	−1.858[a]	0.541	1.72	(1.02–2.90) TU	$P > 0.1$
					EC50		
Body	Cu	110.7	−2.899[a]	3.783	43.9[a]	(32.2–60.0) mg/kg	
	Zn	92.71	−51.05[b]	5.918	372[b]	(361–383) mg/kg	
	Mixture	96.87	−6.457[ab]	0.622	1.86	(1.64–2.11) TU	$P < 0.001$
					EC25		
Body	Cu				30.1	(18.8–48.1) mg/kg	
	Zn				364	(349–379) mg/kg	
	Mixture				1.93	(1.47–2.51) TU	
					EC10		
Body	Cu				20.6	(10.5–40.3) mg/kg	
	Zn				356	(333–380) mg/kg	
	Mixture				1.99	(1.25–3.17) TU	

Note. EC50s for total, soluble, extractable, and body metal concentrations; EC25s and EC10s only for body metal concentrations. Different superscripts in the column for slope parameter b indicate differences between slopes of curves (comparison among curves per exposure measurement, $P < 0.1$). Different superscripts in the column for ECx indicate significant differences in relative toxicity of copper and zinc assessed at the EC50 level ($P < 0.05$). P values in the column for "deviation from CA" indicate (dis)similarity of the mixture effect from concentration additivity at the EC50 level.

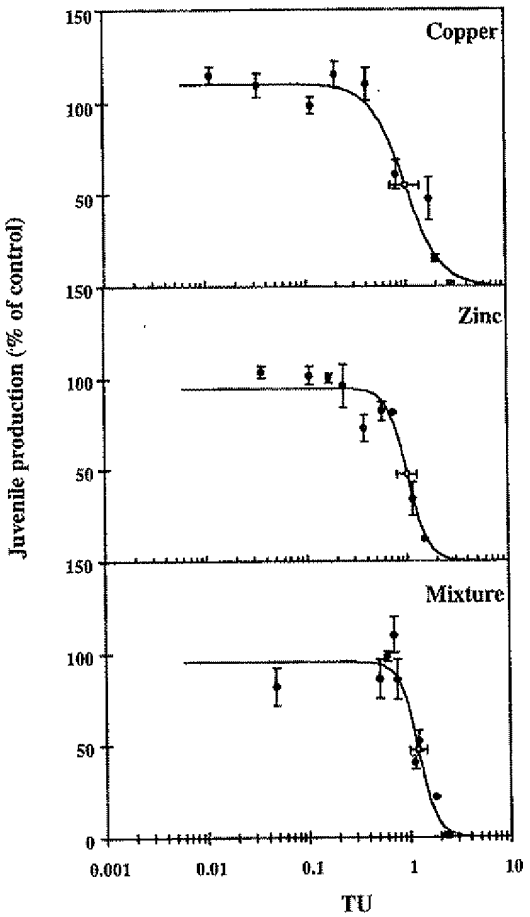

FIG. 1. Concentration–effect relationships for copper, zinc, and mixtures of copper and zinc, on juvenile production in *Enchytraeus crypticus*. Observations (closed circles, with SE) are normalized to the average control reproduction (100%). To illustrate comparison of slopes among curves, curves of copper and zinc separately were also standardized upon their EC50 (by definition 1 TU, open circles, with 95% confidence intervals).

The metals were equally toxic when toxicity is expressed using soluble concentrations, but copper is significantly more toxic when exposure is expressed by extractable and (particularly) body concentrations (Table 2). The effect of the mixture did not differ significantly from the expected value of 1 TU at the level of soluble and extractable concentrations, but was significantly less than concentration additive when exposure was expressed by body concentrations. In the latter case, toxic effects were less than the expected 50% at the mixture concentration of 1 TU, and 50% reduction of juvenile production was reached at a concentration of 1.86 TU.

To extrapolate the findings to effect levels other than 50%, comparisons were made between the slopes of the concentration–effect curves. *F* tests did not demonstrate significant differences among slopes for any of the four exposure measures (all $P > 0.25$ for total, extractable, and soluble concentrations, but $P < 0.1$ between curves for copper and zinc for body concentrations, see Table 2). Similarity of slopes suggests that the type of mixture effect is grossly the same at all concentration levels studied. To investigate this in more detail, TUs were also defined from the EC25 and the EC10 for body concentrations, the measure for which slope divergence was close to significance. This demonstrated that EC25 and EC10 of the mixture in the body tended to increase from 1.86 TU at the EC50 level, via 1.93 TU at the EC25 level, to 1.99 TU at the EC10 level (Table 2). It is furthermore noticed that the confidence interval of EC*x* increased at low effect levels. This suggests that a significant deviation from concentration addition is more difficult to establish at low than at intermediate effect levels.

Metal Availability in Soil

The slopes *n* of the isotherms for copper and zinc added separately were similar for both extraction fluids (Table 3), although for both metals there was a tendency for higher saturation rates of the solid phase in $CaCl_2$ solution than in water. Copper sorbs more strongly to the solid phase than zinc, in particular in the $CaCl_2$ solution for which the sorption parameters K_F differed by a factor of 4.

The slope *n* increased significantly for zinc when copper was added and tended to increase for copper when zinc is added (not significant). The sorption parameter K_F decreased considerably for zinc when copper was added. K_F decreased signifi-

TABLE 3

Parameters of the Freundlich Isotherms (with Back-Transformed Standard Errors, SE, of K_F and *n*) for Metals Applied Singly (S) or in Mixture with the Other (M), Using Water Solubility and 0.01 *M* $CaCl_2$ extractability to Quantify Liquid Phase Concentrations

			Freundlich parameters				
Metal	Extraction	Type	n	(SE)	K_F	(SE)	r^2
Copper	Water	S	1.34 a	(1.03–1.91)	1073 a	(571–2016)	0.92
	Water	M	2.05 a	(1.71–2.57)	1051 b	(847–1303)	0.95
	$CaCl_2$	S	1.89 a	(1.40–2.91)	993 a	(577–1711)	0.92
	$CaCl_2$	M	2.71 a	(2.40–3.12)	926 a	(815–1051)	0.98
Zinc	Water	S	1.19 a	(0.89–1.79)	959 b	(479–1921)	0.90
	Water	M	1.83 b	(1.45–2.45)	212 nd	(158–286)	0.92
	$CaCl_2$	S	1.39 a	(1.19–1.66)	248 c	(210–293)	0.97
	$CaCl_2$	M	2.00 b	(1.75–2.33)	84 nd	(77–92)	0.97

Note. Significant differences among slopes (*n*) or intercepts (K_F), at $P < 0.05$, are indicated by different letters following the parameter. nd, not determined.

cantly for copper when zinc was added when judged by water solubility and tended to decrease when judged by $CaCl_2$ extractability. So, the most dominant effect of copper addition is the higher saturation rate of the solid phase for zinc, together with the decreased sorption of zinc. Zinc addition only slightly increased the solubility of copper, and this effect was only found in water. These findings are evidence for a strong interaction effect of copper on zinc outside the body. Figure 2 illustrates this, demonstrating that addition of copper brings zinc in solution, both in water and in 0.01 *M* $CaCl_2$.

Body Concentrations

Body concentrations increased at increasing exposure concentrations for the metals applied singly (Fig. 3). Background body concentrations of copper and zinc in control conditions were 6.5 mg Cu and 108 mg Zn/kg dry wt. The body concentration of copper was largely unaffected by total soil copper concentrations up to 100 mg/kg, suggesting maintenance of copper homeostasis at low exposure concentrations. For zinc the lowest exposure concentration (either added separately or in mixture) caused increased body concentrations; there is no evidence for regulation of zinc at the exposure levels used in the experiment. At the highest exposure concentration the increase of the body concentration compared to the control was approximately a factor 16 for copper and a factor 5 for zinc.

After exposure to the mixture, body concentrations also increased, but this increase differed from the pattern observed after separate exposures. First, by using total soil concentrations to express exposure, it appeared that uptake of copper was not affected by zinc, whereas uptake of zinc is strongly affected by copper (Fig. 3 and Table 4: slope comparisons). This can be the result of various types of interactions: between the metals among each other and with the soil, during uptake, or both. Second, to unravel this further, soluble and extractable concentrations were used to express exposure. A similar pattern appeared, but with the more precise implication that the uptake of copper from the water-soluble or $CaCl_2$-extractable copper concentration is largely unaffected by zinc, whereas the uptake of zinc from the soluble zinc concentrations increased significantly in the presence of copper. Assuming that exposure is (at least partly) governed by the liquid phase, these results are an indication for the occurrence of interactions during uptake. Third, the concentrations of the metals in the body were summed on the basis of molar concentrations, to assess whether total metal uptake was reduced or stimulated in the mixture. This exercise demonstrated that the total metal concentration in the body of animals exposed to the mixture was lower than expected (Fig. 4), which probably contributed to the less than concentration additive effects observed using body concentrations.

Equitoxicity

The total metal concentrations in soil were not equitoxic in the mixture series, due to variation among EC50s for copper and zinc between the range-finding experiments and the mixture experiment. Equitoxicity would have implied that the concentration ratio of copper and zinc would equal unity at all concentration levels (except the background). In the mixture series, however, the concentration ratio copper/zinc was 3.5 when judged by total soil concentrations. Due to the interactions demonstrated above, the copper/zinc ratio was different for other exposure measures, viz. 2, 0.5, and 0.15 for extract-

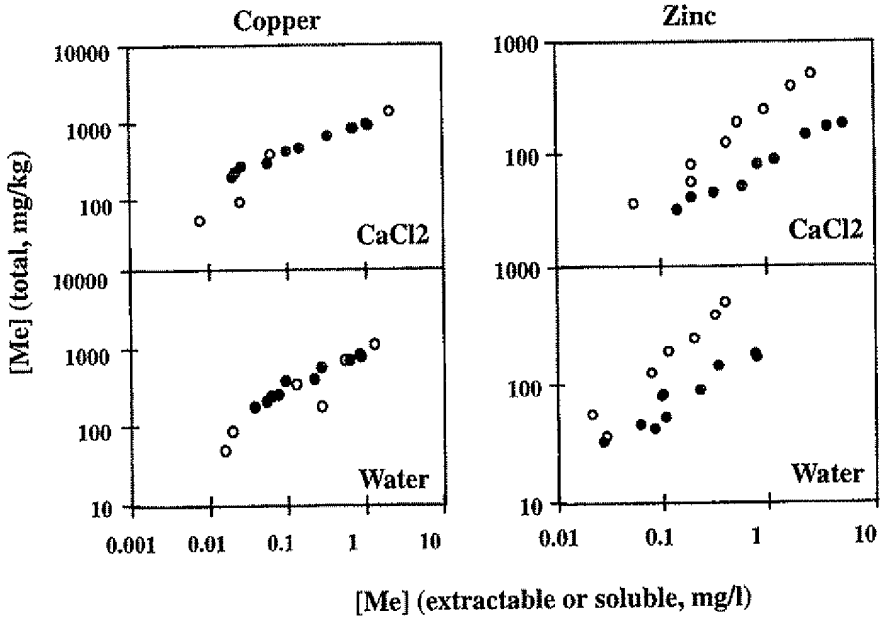

FIG. 2. Freundlich isotherms for copper and zinc in artisoil, based on 0.01 *M* $CaCl_2$ extractable or water soluble metal concentrations (*X*-axis) and total metal concentrations in artisoil (*Y*-axis) in the absence (open circles) and presence (closed circles) of the other metal. Freundlich isotherm parameters are given in Table 3.

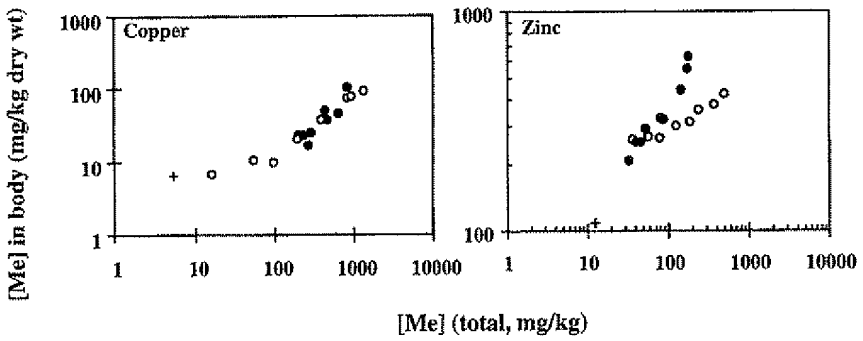

FIG. 3. Relationships between concentrations in soil and in the body of *Enchytraeus crypticus* for copper and zinc after exposure to the metals applied singly (open cirlces) or in mixture with the other (closed circles). Crosses indicate the metal body concentrations under control conditions.

able, soluble, and body concentrations, respectively. These observations indicate a relative excess of copper when toxicity is expressed through total soil concentrations and of zinc when expressed through body concentrations.

DISCUSSION

Methodological Aspects of Study Design and Methods

The need to consider joint toxicity models and bioavailability simultaneously complicates the analysis of joint effect studies with soil organisms. Therefore, prior to comparison of results with other findings, some methodological aspects are evaluated. These aspects relate to variation of sensitivity among experiments, salt effects, the relevance of exposure expressions for "true bioavailability," and the appropriateness of toxicological models when MoA is undefined.

The calculation of toxic units. EC50s for copper and zinc differed among range findings and the mixture study (copper, 873 versus 477 mg Cu/kg soil; zinc, 188 versus 336 mg Zn/kg soil). Variation of sensitivity among experiments after exposure in artisoil has also been found for other species, for example, the worm *Eisenia andrei* (Weltje *et al.,* 1995) and the springtail *Folsomia candida* (Van Gestel and Hensbergen, 1997). Such differences are often attributed to interexperiment variation, caused by, for example, differences among generations. For artisoil pH variation among experiments may be an important cause, since metal sorption to kaolin clay is strongly influenced by slight pH variation in the range between 5 and 7 (Holm and Zhu, 1994). Since uptake is assumed to be governed by liquid phase transport (see below), pH variation may thus strongly influence exposure and effects. Variation among experiments is often neglected due to resource limitation (space, time), joint effects being calculated on the basis of range-finding and literature data. For *E. crypticus* this can also be done. The $TU_{observed}$ of the copper + zinc mixture calculated from range-finding results appeared to be 0.2 TU lower than

TABLE 4
Parameters of Linear Regression Lines between Log-Body Concentration of Metals and Log-Metal Concentrations in Soils, Total (mg/kg), Water-Soluble (mg/L), or 0.01 *M* $CaCl_2$- extractable (mg/L)

Metal	Extraction	Type	Slope	95% CI	Intercept	SE	r^2
Copper	Total	S	0.663 a	(0.507–0.820)	−0.122 a	(−0.503–0.260)	0.947
	Total	M	1.03 a	(0.507–1.553)	−1.107 a	(−2.458–0.244)	0.795
	Water	S	0.510 a	(0.354–0.665)	1.900 a	(1.711–2.089)	0.915
	Water	M	0.543 a	(0.243–0.843)	2.05 a	(1.747–2.353)	0.766
	$CaCl_2$	S	0.412 a	(0.250–0.575)	1.902 a	(1.694–2.110)	0.895
	$CaCl_2$	M	0.412 a	(0.232–0.592)	1.988 a	(1.771–2.205)	0.839
Zinc	Total	S	0.185 a	(0.134–0.236)	2.102 nd	(1.990–2.214)	0.929
	Total	M	0.553 b	(0.438–0.668)	1.488 nd	(1.269–1.708)	0.949
	Water	S	0.154 a	(0.079–0.228)	2.651 nd	(2.572–2.731)	0.809
	Water	M	0.314 b	(0.243–0.384)	2.783 nd	(2.718–2.848)	0.940
	$CaCl_2$	S	0.135 a	(0.095–0.175)	2.545 nd	(2.521–2.570)	0.919
	$CaCl_2$	M	0.280 b	(0.221–0.339)	2.552 nd	(2.521–2.583)	0.947

Note. Significant differences among slopes or intercepts, at $P < 0.05$, are indicated by different letters following the parameter. nd, not determined.

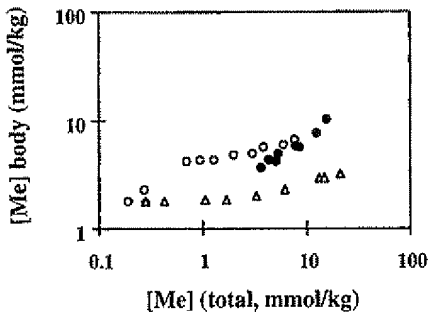

FIG. 4. Relationship between summed copper and zinc concentrations in soil and in the body of exposed *Enchytraeus crypticus* (summation on the basis of molar concentrations) for the animals exposed to zinc only (open circles), copper only (triangles), or the mixture (closed circles).

the value based on simultaneously collected toxicity data for copper and zinc (Table 2, total soil concentrations). This difference is small, which is probably due to the increased sensitivity for copper and the decreased sensitivity for zinc between the range-finding and mixture experiments. The effect of sensitivity variation may, however, be larger. Van Gestel and Hensbergen (1997) found a difference of approximately 0.5 TU between both methods for the joint effect of cadmium and zinc in the springtail *F. candida*. Although the difference between both calculation methods may be incidentally small, it is evident that conclusions based on simultaneously collected separate and joint toxicity data are more reliable since undesired error is reduced.

Effects related to anion balance. Balance of chloride through addition of sodium chloride has various implications. First, the additions caused a slight sodium unbalance, with 1426 mg Na/kg in the control versus a background of 368 mg Na/kg in the treatment with the highest metal concentration. The sodium effect cannot be assessed, since the availability of indigenous and added sodium is unknown. The potential effect is, however, probably negligible in comparison to that of the metals. Second, direct toxic effects of chloride may occur. These cannot be neglected, since chloride was found to have affected juvenile production in the experiment. Third, chloride may also indirectly affect reproduction through its influence on metal speciation (*e.g.,* Boekhold, 1992; Chardon, 1984; Babich and Stotzky, 1978): ionic metal species will dominate at low chloride concentrations, while metal–chloride complexes will dominate at high chloride concentrations. This indirect effect relates to metal exposure, since the uptake of metal–chloride complexes needs not be similar to the uptake of ionic metal. Although chloride effects thus certainly have occurred during exposure, the additional observations on sorption and uptake were made to take the indirect chloride effects into account. When exposure is expressed using soluble, extractable, and body concentrations, it may be assumed that the indirect effects of chloride are neutralized. Thus, salt stress in *E. crypticus* may have directly affected reproductive output numerically, but it is likely that this may bias only the conclusion based on total metal concentrations in soil.

Relevance of exposure expressions. The free metal ion is often considered to be the metal species actually taken up by plants (*e.g.,* Sposito 1984), and exposure of aquatic organisms has been related to both the free metal ion and the metal hydroxide concentration in the water phase (Newman and Jagoe, 1994). Despite this, interactions among metals and with soil were studied with extraction-based methods (*e.g.,* Gupta and Aten, 1993), since metal speciation in soil can presently not be quantified routinely (Ure and Davidson, 1995). It may be debated whether interactions found by extraction methods are related to "true bioavailability," since there is, for example, evidence that ingestion through food is also a relevant uptake route of toxic ions in earthworms (*e.g.,* Janssen, M.P.M., *et al.,* 1996). Recent uptake and toxicity studies found, however, that water- or neutral salt-extractable metal concentrations are considerably better related to uptake and effects of metals in earthworms than total soil concentrations (Janssen, R.P.T., *et al.,* 1996, Spurgeon and Hopkin, 1996; Posthuma and Notenboom, 1996). Therefore, it is likely that the interactions between copper and zinc that were demonstrated with the extraction-based methods have indeed influenced the joint effects of copper and zinc in the enchytraeid.

Interactions, mode of action and toxicological models. The joint effects of copper and zinc were assessed in comparison to predictions from SSA. For metals, it can be debated whether SSA is appropriate. First, the assumption of "no interaction" may be violated. Metal–metal interactions, such as precipitation among some pairs of metal ions or competitive displacement at sorption sites, are well known (*e.g.,* Magos and Webb, 1978). Competitive displacement may occur in soil and during uptake (see below) and may be the principal type of process that modulates the joint effects at the target site. Indirect interactions may also occur, like the synthesis of specific metal binding proteins when they are activated by another metal (*e.g.,* Willuhn *et al.,* 1996; Dallinger, 1996). Second, the assumption of similar MoA's may be invalid. Some metals have a metabolic function in metalloenzyme complexes. Shortage of such metals in the diet may increase toxicity of other metals (*e.g.,* Medici and Taylor, 1967). In particular at the concentration of homeostatic regulation, metals with the function of micronutrient have highly specific metabolic functions. Thus, both assumptions of SSA may not hold for metal mixtures.

Comparison of observed joint effects with the predictions of various null models has been suggested as solution for cases in which the appropriateness of a single null model may be debated (Vranken *et al.,* 1988). In addition to comparison with SSA, it is numerically possible to compare observations to the prediction from independent dissimilar action (hereafter referred to as IDA; defined for mixtures with no interaction and dissimilar MoA's). The joint effect related to IDA is response addition (assuming full positive correlation of sensitivities to

the chemicals, see Hewlett and Plackett (1959)). This approach was not applied, however, since SSA and IDA are quantitatively related (Drescher and Boedeker, 1995). This means that, for example, an observed joint effect of a binary mixture at $TU_{observed} = 1.5$ can equally be quoted as "antagonism" in comparison to concentration addition (expectation, 1 TU) and as "synergism" compared to response addition (expectation: 2 TU).

The debate on the choice of null models remains unsolved, unless mechanistic information is available for a particular mixture. The SSA model was used as a method to quantify the joint effect. Findings thus do not imply that "true SSA" has occurred at the level of total soil concentrations. On the contrary, the similarity of the response to concentration addition at the level of total soil concentrations was found to be affected by nontoxicological interactions outside the body.

Sorption, uptake and effects of metal mixtures

Interactions in soil. Interactions of chemicals with soil and among each other are of prime importance for exposure assessment of separate chemicals (Dickson *et al.,* 1994) and mixtures (Calamari and Vighi, 1992). Sorption has been studied usually for metals separately. For zinc, the partition coefficient between solid and liquid phase (neutral salt extraction) varied between approximately 2 and 800 L/kg for field soils to which zinc was added (Buchter *et al.,* 1989). In field soils with moderate (mixed) metal pollution the partition coefficient of zinc (based on pore water concentrations) varied between 6 and 6700 L/kg (Janssen, R.P.T., *et al.,* 1996b). For copper applied singly the variation ranged from approximately 50 to 6400 L/kg and for contaminated field soils from 25 to 4300 L/kg. Van Gestel and Hensbergen (1997) found a value of 463 L/kg for zinc in artisoil (based on water soluble concentrations). In the present experiment with artisoil, the partition coefficients were 248 L/kg for zinc and 993 L/kg for copper (based on neutral salt-extractable concentrations) and 959 resp. 1073 L/kg (based on soluble concentrations). These values are within the range found for field soils, although comparisons are complicated by different expressions of the liquid phase concentrations and by a slight overestimation of the artisoil values due to the small increase of acidity during the enchytraeids exposure. The sorption of metals in artisoil is, however, not peculiarly low or high in comparison to field soils. The variation of sorption strength among field soils indicates, however, that soil type has a major influence on sorption.

The results revealed interactions among metals in artisoil, *viz.* copper reduced sorption of zinc in artisoil. Some studies confirm the occurrence of interactions among metals also for field soils. For example, Luo and Rimmer (1995) reported that copper increased the extractability of zinc, whereas zinc addition hardly affected copper extractability. Chardon (1984) demonstrated that an excess of zinc or copper increased the extractability of cadmium. The latter interaction could not be demonstrated at low concentrations, but it is unknown whether this implies lack of interaction at the level of true bioavailability.

For prediction of interactions among metals in field soils, it is important to note that sorption can be grossly predicted on the basis of soil characteristics (Janssen, R.P.T., *et al.,* 1996b; Sheppard and Thibault, 1990, and references therein). The presence of a soil type dependent but grossly fixed sorption sequence of metal ions may imply that the effects of sorption on joint effects may be in part predictable. The sequence of the relative sorption strength is important for such a prediction, in relation to the relative toxicities of the metals. If there is a sorption sequence and also a difference in relative toxicities, then the outcome of TUs based on total concentrations will differ from that based on concentrations in which availability is taken into account, *viz.* the soluble, extractable, or body concentrations. For example, in the present study it was found that the influence of relative toxicities and solubilities caused a difference between joint effects assessed by body concentrations ($TU_{observed}$ less than $TU_{expected}$) and total soil concentrations ($TU_{observed}$ grossly similar to $TU_{expected}$), with copper being more toxic than zinc and zinc being more soluble. The joint effects assessed by total soil concentrations were thus qualified as more severe than when judged by body concentrations. Both the expected and the observed EC50s are influenced by this. For other metal pairs the balance of both sequences may work out differently. It can be hypothesized that an increased solubility of the most toxic metal due to the presence of another metal will cause the opposite effect, *viz.* joint effects assessed by total concentrations are less severe than when assessed by soluble, extractable, or body concentrations. The sorption data summarized above suggest that interactions in soil may modulate joint toxic effects of chemicals in the body. The quantitative role of this factor should be judged further on the basis of additional data.

Interactions during uptake. Next to the recognition of fixed sequences for (interactive) sorption of metals to soil, it may also be possible to recognize patterns for interactions between metals at the level of uptake in organisms. In *E. crypticus* indications were found for interactions between copper and zinc during uptake, based on the assumption that soluble or extractable concentrations are related to true bioavailability. Mutual influences between metals at the level of body concentrations were also found by other authors, both for animals and plants. For example, Ireland and Fischer (1978) found that lead reduced iron concentrations in the earthworm *Lumbricus terrestris.* Vogel (1988) found that cadmium increased zinc concentrations in the beetle *Tenebrio molitor.* Van Capelleveen (1987) found that cadmium and zinc increased each others concentrations in the isopod *Porcellio scaber.* Migula *et al.* (1989a,b) demonstrated that zinc decreased lead and cadmium concentrations in the cricket *Acheta domesticus.* McKenna *et al.* (1993) found that cadmium increased uptake of zinc in lettuce but not in spinach, while in both plants zinc influenced

cadmium uptake in a concentration-dependent way. Finally, Luo and Rimmer (1995) found that copper increased zinc concentrations in the plant *Hordeum vulgare*. In the last example, the uptake pattern was associated to the interaction pattern found in soil using extraction techniques. Other authors found no interaction at the level of body concentrations, for example, Berger *et al.* (1994) (zinc and cadmium in the snail *Helix pomatia*) and Van Gestel and Hensbergen (1997) (cadmium and zinc in the springtail *F. candida*).

Recognition of interaction sequences in body concentration data is more difficult than for soil only. First, interactions at the level of body concentrations have often been studied without distinguishing competitive displacement in soil from interactions during the uptake process. Second, species-specific characteristics may play a role. Berger *et al.* (1994), for example, found that zinc and cadmium were sequestered in different compartments of the body of the snail *H. pomatia*. This suggests that interaction among binding sites in the organism is minimized. Beeby (1991) gives more examples of species-specific mechanisms that may promote or reduce interactions among metals. Interactions during uptake are thus apparently influenced by species-specific mechanisms, and these may be neutral or increase or reduce joint effects.

Joint toxicity data for soil organisms. In the present study a less than concentration additive effect of a mixture of copper and zinc in the body of *E. crypticus* was demonstrated next to modulation of this joint toxic effect by interactions in soil and during uptake. The joint effect of cadmium + zinc for *E. crypticus,* and of cadmium + zinc, cadmium + copper, and zinc + copper for the compost worm *E. andrei* was previously studied, applying the same null model. Grossly less than concentration additive effects were found (Posthuma *et al.,* 1995; Weltje *et al.,* 1995). A less than concentration additive effect was also demonstrated on reproduction in the worm *Aporrectodea caliginosa* exposed to the tertiary mixture of cadmium, copper, and zinc (Khalil *et al.,* 1996). Previously found was a less than concentration additive effect of cadmium + pyrene in the compost worm (Posthuma *et al.,* 1996), chemicals for which different MoA's are evident. Van Gestel and Hensbergen (1997) studied the joint effects of cadmium + zinc in the springtail *F. candida* and demonstrated a less than concentration additive effect in the body that is moderated by interactions in soil. Direct interactions in soil could, however, not be demonstrated with extraction-based methods for all pairs of chemicals in previous studies with earthworms. Nonetheless, the joint (toxicological) effect in the bodies of all studied soil invertebrates appeared to be moderated by sorption, since in all cases the divergence from SSA depended on the expression of exposure used to calculate TUs.

Comparison to SSA has not been the only method to analyze joint effects of metals in soil organisms. Various authors interpreted significant two-way interaction in ANOVA as evidence for "synergism" or "antagonism" (*e.g.,* Medici and Taylor, 1967; Doelman *et al.,* 1984; Van Capelleveen, 1987; Vogel, 1988; Migula, 1989; Migula *et al.,* 1989a,b). This has been criticized by Michaud *et al.* (1994), since this method is appropriate only when concentration–effect relationships are linear. If the curve is nonlinear, then positive two-way interaction is to be expected due to the fast sigmoidal increase of response just beyond NOEC, which falsely suggests "more than concentration additivity," whereas at high concentrations the opposite will occur. Numerical evidence for this is given by Hensbergen and Van Gestel (1995); ANOVA demonstrated synergism of cadmium and zinc in the isopod *P. scaber* (data from Van Capelleveen, 1987), whereas TU calculations indicated a response similar to concentration additivity ($TU_{observed}$ approx. 1.1). Nonetheless, ANOVA may be helpful in mixture analyses to demonstrate whether a chemical contributes to toxicity. For example, Laskowski and Hopkin (1996) compared EC50 values of zinc in the presence and absence of three other metals for the snail *H. aspersa*. The toxic effect of the mixture was stronger than that of zinc alone. In that case, the other metals evidently contributed to toxicity, but the type of joint effect caused by the mixture could not be identified.

The few studies on joint metal effects in soil organisms tend to indicate a response that varies between "similar to concentration additive" and "less than concentration additive" ($TU_{observed}$ based on total soil concentrations varies between approx. 1 and 2). This conclusion is based, however, on observations at the level of overt toxic effects, mostly at the EC50 level. Also investigated, however, was whether joint effects are different at low concentration levels. In the field, concentrations are lower than the concentrations applied in the experiment, and they are rarely (if ever) equitoxic. The calculations indicated that the $TU_{observed}$ for *E. crypticus* tended to increase between EC50 and EC10, suggesting a tendency for response addition (assuming full correlation of sensitivities). This indicates a tendency toward less severe effects than expected from SSA at low exposure concentrations. Wallace and Berry (1989) and Berry and Wallace (1989) studied interactive effects of various metal mixtures in lettuce. Their data also suggest that the joint effect of two or three metals below threshold concentrations tend to be response additive, whereas at toxic mixture concentrations the observed effects are often stronger than expected from response addition. Referring to the discussion of alternate null models, it is possible that for low exposure concentrations response addition (with full correlation of sensitivities for both metals) may be better justified as null model than concentration addition. Probably, at low concentrations, copper and zinc have different MoA's, related to their specific roles as micronutrients. Van Gestel and Hensbergen (1997), however, found the joint effects of cadmium and zinc on the springtail *F. candida* tended toward concentration addition at low exposure concentrations.

The few available data indicate that mixture effects of metals at low concentrations differ between studies, *viz.* tendencies toward concentration and response additivity were both found.

For chemicals with similar MoA's, concentration addition is expected to apply at all concentration levels (see, *e.g.,* Deneer *et al.,* 1988; Warne and Hawker, 1995). For metals with probably different MoA's, however, such a generalization cannot be made at present. Nonetheless, concentration dependency of joint effects may be important for assessing the environmental risks of chemicals with different MoA's. *How* concentration dependency depends on the characteristics of particular mixtures can, however, only be solved with additional data.

CONCLUSIONS

Implications for Risk Assessment

Both concentration addition, based on SSA, and response addition, based on IDA, are operational models that can be applied in a quantitative way in regulatory frameworks for risk assessment and derivation of environmental quality objectives. Concentration addition is an obvious choice when considering mixtures of chemicals that have a similar MoA and that do not interact. For mixtures of chemicals with unknown MoA's, the choice between alternate null models can be disputed. This may cause the validity of risk assessments based on them to be disputable. It is difficult to solve this debate on appropriateness of null models on the basis of quantitative data, since concentration and response addition often fit equally well to the data, in particular, at low (environmentally relevant) concentrations (Drescher and Boedeker, 1995). From the viewpoint of environmental protection, however, preference for SSA as an operational model for mixtures with unknown MoA's and interaction types may be defensible, since SSA is relatively more precautious than IDA. This choice would be unjustified when joint effects are strongly—or more frequently—synergistic or antagonistic in comparison to SSA or when the alternate null model is better justified for a particular mixture. For organisms exposed to metal mixtures in water, joint effects of mostly binary and tertiary mixtures are in the range of 0.5 to 2 TU and approximately equally distributed between SSA and responses that are synergistic or antagonistic in comparison to this (*e.g.,* Enserink *et al.,* 1991; Kraak *et al.,* 1992, 1993, 1994, and references therein). This pattern may reflect true toxicological interactions inside the bodies of the organisms, since the metal fraction sorbed to particles is low in aquatic media (Enserink *et al.,* 1991). For soil ecotoxicological risk assessment it should be investigated whether the joint effect pattern found in aquatic studies is moderated systematically by sequences of sorption strengths in soil and relative toxicities. The present study demonstrated that in soil the interactions among the chemicals outside the body may be important, but also demonstrated that additional data are needed to formulate generalized conclusions.

ACKNOWLEDGMENTS

This study was supported by the Dutch Ministry of Housing, Spatial Planning and the Environment. Collegues at the Laboratory for Ecotoxicology as well as at the Department of Ecology and Ecotoxicology at the Vrije Universiteit of Amsterdam, The Netherlands, provided a welcome forum to discuss the research topic. Kees van Gestel and Paul Hensbergen (Vrije Universiteit, Amsterdam) and Lennart Weltje and Jos Notenboom (RIVM) will recognize their valuable contributions to the formulation of the ideas presented here. Arthur De Groot is acknowledged for the implementation of the micromethods for metal analyses in enchytraeids and Jeroen Agterberg, Marcel Ott, and Carlo Strien are acknowledged for metal measurements in soils and extracts. Abdi Kalif performed the required range-finding experiments.

REFERENCES

Arnold, S. F., Klotz, D. M., Collins, B. M., Vonier, P. M., Guillette, L. J., and McLachlan, J. A. (1996). Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **272,** 1489–1492.

Babich, H., and Stotzky, G. (1978). Toxicity of zinc to fungi, bacteria, and coliphages: Influence of chloride ions. *Appl. Environ. Microbiol.* **36,** 906–914.

Beeby, A. (1991). Toxic metal uptake and essential metal regulation in terrestrial invertebrates: A review. In *Metal Ecotoxicology: Concepts and Applications* (M. C. Newman and A. W. McIntosh, Eds.), pp. 65–89. Lewis, Boca Raton, FL.

Berger, B., Dallinger, R., Gruber, A., and Moser, J. (1994). Uptake, assimilation, and ligand binding of cadmium and zinc in *Helix pomatia* after combined exposure to both metals. In *Ecotoxicology of Soil Organisms.* (M. H. Donker, H. J. P. Eijsackers, and F. Heimbach, Eds.), pp. 347–354. Lewis, Boca Raton, FL.

Berry, W. L., and Wallace, A. (1989). Interaction of the yield response surface of lettuce with high and toxic concentrations of zinc and nickel. *Soil Sci.* **147,** 398–400.

Boekhold, A. E. (1992). *Field Scale Behaviour of Cadmium in Soil.* Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Buchter, B., Davidoff, B., Amacher, M. C., Hinz, C., Iskandar, K., and Selim, H. M. (1989). Correlation of Freundlich K_d and n retention parameters with soils and elements. *Soil Sci.* **148,** 370–379.

Calamari, D., and Alabaster, J. S. (1980). An approach to theoretical models in evaluating the effects of mixtures of toxicants in the aquatic environment. *Chemosphere* **9,** 533–538.

Calamari, D., and Vighi, M. (1992). A proposal to define quality objectives for aquatic life for mixtures of chemical substances. *Chemosphere* **25,** 531–542.

Chardon, W. J. (1984). *Mobility of Cadmium in Soils.* Thesis, Agricultral University, Wageningen, The Netherlands.

Dallinger, R. (1996). Metallothionein research in terrestrial invertebrates: Synopsis and Perspectives. *Comp. Biochem. Physiol. C* **113,** 125–133.

De March, B. G. E. (1987). Simple similar action and independent joint action—Two similar models for the joint effects of toxicants applied as mixtures. *Aquat. Toxicol.* **9,** 291–304.

Deneer, J. W., Sinnige, T. L., Seinen, W., and Hermens, J. L. M. (1988). The joint toxicity to *Daphnia magna* of industrial organic chemicals at low concentrations. *Aquat. Toxicol.* **12,** 33–38.

Dickson, K. L., Giesy, J. P., and Wolfe, L. (1994). Summary and conclusions. In: *Bioavailability: Physical, Chemical and Biological Interactions* (J. L. Hamelink, P. F. Landrum, H. L. Bergman, and W. H. Benson, Eds.), pp. 221–230. CRC Press, Boca Raton, FL.

Dirven-Van Breemen, E. M., Baerselman, R., and Notenboom, J. (1994). *Onderzoek naar de geschiktheid van de potwormsoorten* Enchytraeus albidus *en* E. crypticus (*Oligochaeta, Annelida*) *in bodemecotoxicologisch onderzoek,* Report No. 719102025. RIVM, Bilthoven, The Netherlands.

Doelman, P., Nieboer, G., Schrooten, J., and Visser, M. (1984). Antagonistic and synergistic toxic effects of lead and cadmium in a simple food chain: Nematodes feeding on bacteria or fungi. *Bull. Environ. Contam. Toxicol.* **32,** 717–723.

Drescher, K., and Boedeker, W. (1995). Assessment of the combined effects of substances: the relationship between concentration addition and independent action. *Biometrics* **51,** 716–730.

Enserink, E. L., Maas-Diepeveen, J. L., and Van Leeuwen, C. J. (1991). Combined effects of metals: An ecotoxicological evaluation. *Wat. Res.* **25,** 679–687.

Gupta, S. K., and Aten, C. (1993). Comparison and evaluation of extraction media and their suitability in a simple model to predict the biological relevance of heavy metal concentrations in contaminated soils. *Int. J. Environ. Anal. Chem.* **51,** 25–46.

Haanstra, L., Doelman, P., and Oude Voshaar, J. H. (1985). The use of sigmoidal dose response curves in soil ecotoxicological research. *Plant Soil* **84,** 293–297.

Hensbergen, P. J., and Van Gestel, C. A. M. (1995). Technische Commissie Bodembescherming, TCB Report No. R04(1995) Den Haag, The Netherlands.

Hewlett, P. S., and Plackett, R. L. (1959). A unified theory for quantal responses to mixtures of drugs: Non-interactive action. *Biometrics* **15,** 591–610.

Holm, T. R., and Zhu, X.-F. (1994). Sorption by kaolinite of Cd2+, Pb2+ and Cu2+ from landfill leachate-contaminated groundwater. *J. Contamin. Hydrol.* **16,** 271–287.

Ireland, M. P., and Fischer, E. (1978). Effect of Pb^{++} on Fe^{+++} tissue concentrations and delta-aminolaevulinic acid dehydratase activity in *Lumbricus terrestris*. *Acta Biol. Acad. Sci. Hung.* **29,** 395–400.

Janssen, M. P. M., Glastra, P., and Lembrechts, J. F. M. M. (1996). Uptake of ^{134}Cs from a sandy soil by two earthworm species: the effect of temperature. *Arch. Environ. Contam. Toxicol.* **31,** 184–191.

Janssen, R. P. T., Peijnenburg, W. J. G. M., Posthuma, L., and Van Den Hoop, M. A. G. T. (1997a). Equilibrium partitioning of heavy metals in Dutch field soils. I. Relationships between metal partition coefficients and soil characteristics. *Environ. Toxicol. Chem.*, in press.

Janssen, R. P. T., Posthuma, L., Baerselman, R., Den Hollander, H. A., Van Veen, R. P. M., and Peijnenburg, W. J. G. M. (1996a). *Equilibrium Partitioning of Heavy Metals in Dutch Soils: Prediction of Metal Accumulation in Earthworms.* Report No. 719101028. RIVM, Bilthoven, The Netherlands.

Janssen, R. P. T., Posthuma, L., Baerselman, R., Der Hollander, H. A., Van Veer, R. P. M., and Peijnenburg, W. J. G. M. (1997b). Equilibrium partitioning of heavy metals in Dutch field soils. II. Predictions of metal accumulation in earthworms. *Environ. Toxicol. Chem.*, in press.

Janssen, R. P. T., Pretorius, P. J., Peijnenburg, W. J. G. M., and Van Den Hoop, M. A. G. T. (1996b). *Determination of Field-Based Partition Coefficients for Heavy Metals in Dutch Soils and Their Relationships with Soil Characteristics.* Report No. 719101023. RIVM, Bilthoven, The Netherlands.

Khalil, M. A., Abdel-Lateif, H. A., Bayoumi, B. M., Van Straalen, N. M., and Van Gestel, C. A. M. (1996). Effects of metals and their mixtures on survival and cocoon production of the earthworm *Aporrectodea caliginosa*. *Pedobiologia* **40,** 548–556.

Kraak, M. H. S., Lavy, D., Peeters, W. H. M., and Davids, C. (1992). Chronic ecotoxicity of copper and cadmium to the zebra mussel *Dreissena polymorpha*. *Arch. Environ. Contam. Toxicol.* **23,** 363–369.

Kraak, M. H. S., Lavy, D., Schoon, H., Toussaint, M., Peeters, W. H. M., and Van Straalen, N. M. (1994). Ecotoxicity of mixtures of metals to the zebra mussel *Dreissena polymorpha*. *Environ. Toxicol. Chem.* **13,** 109–114.

Kraak, M. H. S., Schoon, H., Peeters, W. H. M., and Van Straalen, N. M. (1993). Chronic ecotoxicity of mixtures of Cu, Zn and Cd to te zebra mussel *Dreissena polymorpha*. *Ecotoxicol. Environ. Saf.* **25,** 315–327.

Laskowski, R., and Hopkin, S. P. (1996). Effect of Zn, Cu, Pb, and Cd on fitness in snails (*Helix aspersa*). *Ecotoxicol. Environ. Saf.* **34,** 59–69.

Luo, Y., and Rimmer, D. L. (1995). Zinc–copper interaction affecting plant growth on a metal-contaminated soil. *Environ. Pollut.* **88,** 79–83.

Magos, L., and Webb, M. (1978). Theoretical and practical considerations on the problem of metal–metal interaction. *Environ. Health Perspect.* **25,** 151–154.

McKenna, I. M., Chaney, R. L., and Williams, F. M. (1993). The effects of cadmium and zinc interactions on the accumulation and tissue distribution of zinc and cadmium in lettuce and spinach. *Environ. Pollut.* **79,** 113–120.

Medici, J. C., and Taylor, M. W. (1967). Interrelationships among copper, zinc and cadmium in the diet of the confused flour beetle. *J. Nutr.* **93,** 307–309.

Michaud, J. P., Gandolfi, A. J., and Brendel, K. (1994). Minireview—Toxic responses to defined chemical mixtures: Mathematical models and experimental designs. *Life Sci.* **55,** 635–651.

Migula, P. (1989). Combined and separate effects of cadmium, lead and zinc on respitatory metabolism during the last larval stage of the house cricket, *Acheta domesticus*. *Biologia* (*Bratislava*) **44,** 513–521.

Migula, P., Kafel, A., Kedziorski, A., and Makonieczny, M. (1989a). Combined and separate effects of cadmium, lead and zinc on growth and feeding in the house cricket (*Acheta domesticus*). *Biologia* (*Bratislava*) **44,** 911–921.

Migula, P., Kedziorski, A., Makonieczny, M., and Kafel, A. (1989b). Combined and separate effects of heavy metals on energy budgets and metal balances in *Acheta domesticus*. *Uttar Pradesh J. Zool.* **9,** 140–149.

Newman, M. C., and Jagoe, C. H. (1994). Inorganic toxicants. In: *Bioavailability: Physical, Chemical and Biological Interactions.* (J. L. Hamelink, P. F. Landrum, H. L. Bergman, and W. H. Benson, Eds.), pp. 39–71. CRC Press, Boca Raton, FL.

OECD. (1984). *Guideline for Testing Chemicals. 207. Earthworm Acute Toxicity Tests,* Adopted April 4, 1984.

Posthuma, L., and Notenboom, J. (1996). *Toxic Effects of Heavy Metals in Three Worm Species* (Eisenia andrei, Enchytraeus crypticus, *and* Enchytraeus albidus: *Oligochaeta*) *Exposed in Artificially Polluted Soil Substrates and Contaminated Field Soils,* Report No. 719102048. RIVM, Bilthoven, The Netherlands.

Posthuma, L., Weltje, L., and Antón-Sanchez, F. A. (1996). *Joint Toxic Effects of Cadmium and Pyrene on Reproduction and Growth of the Earthworm* Eisenia andrei. Report No. 607506001. RIVM, Bilthoven, The Netherlands.

Posthuma, L., Weltje, L., and Notenboom, J. (1995). Mixtures of toxicants and their effects on soil animals. In *RIVM Annual Scientific Report 1994, pp. 137–138. Bilthoven, The Netherlands.*

Sheppard, M. I., and Thibault, D. H. (1990). Default soil solid/liquid partition coefficients, K_ds, for four major soil types: A compendium. *Health Phys.* **59,** 471–482.

Sokal, R. R., and Rohlf, F. J. (1981). *Biometry. The Principles and Practice of Statistics in Biological Research,* 2nd ed. Freeman, New York.

Sposito, G. (1984). The future of an illusion: Ion activities in soil solutions. *Soil Sci. Soc. Am. J.* **48,** 531–536.

Sprague, J. B. (1970). Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. *Water Res.* **4,** 3–32.

Spurgeon, D. J., and Hopkin, S. P. (1996). Effects of variations of the organic matter content and pH of soils on the availability and toxicity of zinc to the earthworm *Eisenia fetida*. *Pedobiologia* **40,** 80–96.

Spurgeon, D. J., Hopkin, S. P., and Jones, D. T. (1994). Effects of cadmium, copper, lead, and zinc on growth, reproduction, and survival of the earthworm *Eisenia fetida* (Savigny): Assessing the enviromental impact of point-source metal contamination in terrestrial ecosystems. *Environ. Pollut.* **84,** 123–130.

Ure, A. M., and Davidson, C. M. (1995). *Chemical Speciation in the Environment.* Chapman & Hall, London.

Van Capelleveen, H. E. (1987). *Ecotoxicity of Heavy Metals for Terrestrial Isopods.* Thesis, Vrije Universiteit, Amsterdam, The Netherlands.

Van Gestel, C. A. M., and Hensbergen, P. J. (1997). Combined toxicity of cadmium and zinc for *Folsomia candida* Willem (Collembola: Isotomidae) in relation to bioavailability in soil. *Environ. Toxicol. Chem.*, **16,** 1177–1186.

Van Straalen, N. M., and Denneman, C. A. J. (1989). Ecotoxicological evaluation of soil quality criteria. *Ecotoxicol. Environ. Saf.* **18,** 241–251.

Van Straalen, N. M., and Løkke, H. (1997). *Ecological Principles for Risk Assessment of Contaminants in Soil.* Chapman & Hall, London/New York.

Van Straalen, N. M., and Van Wensem, J. (1986). Heavy metal content of forest litter arthropods as related to body-size and trophic level. *Environ. Pollut. A* **42,** 209–221.

Vogel, W. R. (1988). Zur Aufnahme und Auswirking der Schwermetalle Zink und Cadmium beim Mehlkäfer *Tenebrio molitor* L. (Col., Tenebrionidae) unter berücksichtigung möglicher Wechselwirkungen. *Zool. Anz.* **220,** 25–32.

Vranken, G., Tire, C., and Heip, C. (1988). The toxicity of paired metal mixtures to the nematode *Monhystera disjuncta* (Bastian 1865). *Marine Environ. Res.* **26,** 161–179.

Wagner, C., and Løkke, H. (1991). Estimation of ecotoxicological protection levels from NOEC toxicity data. *Water Res.* **28,** 1237–1242.

Wallace, A., and Berry, W. L. (1989). Dose–response curves for zinc, cadmium, and nickel in combinations of one, two, or three. *Soil Sci.* **147,** 401–410.

Warne, M. S. J., and Hawker, D. W. (1995). The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: the funnel hypothesis. *Ecotoxicol. Environ. Saf.* **31,** 23–28.

Weltje, L., Posthuma, L., Mogo, F. C., Dirven-Van Breemen, E. M., and Van Veen, R. P. M. (1995). *Toxische Effecten van Mengsels van Cadmium, Zink en Koper op Terrestrische Oligochaeten in Relatie tot Bodem–Chemische Interacties.* Report No. 719102043. RIVM, Bilthoven, The Netherlands. [in Dutch]

Westheide, W., and Graefe, U. (1992). Two new terrestrial *Enchytraeus* species (Oligochaeta, Annelida). *J. Natur. Hist.* 479–488.

Willuhn, J., Otto, A., Koewius, H., and Wunderlich, F. (1996). Subtoxic cadmium concentrations reduce copper toxicity in the earthworm *Enchytraeus buchholzi. Chemosphere* **32,** 2205–2210.

Zar, J. H. (1984). *Biostatistical analysis,* 2nd ed. Prentice-Hall, London.

EXHIBIT C

Arch. Environ. Contam. Toxicol. 24, 16–20 (1993)

ARCHIVES OF
Environmental
Contamination
and Toxicology

The Growth Response of the Green Alga *Chlorella vulgaris* to Combined Divalent Cation Exposure

Joseph W. Rachlin and Albania Grosso

Department of Biological Sciences, Lehman College of the City University of New York, Bedford Park Boulevard West, Bronx, New York 10468-1589, USA

Abstract. Using the growth response of the green alga *Chlorella vulgaris* as a model system, the effects of combinations of the environmentally active cations Cd, Co, and Cu were evaluated. The 96-h static EC_{50} for these cations to *C. vulgaris* were, respectively, 0.89 μM, 9.0 μM, and 2.8 μM, yielding a toxicity series such that Cd > Cu > Co. The cation combinations of Cd + Cu, and Cu + Co acted synergistically, while Cd + Co, and the tri-metallic combination Cd + Cu + Co resulted in antagonistic interactions. Examination of these toxic combinations at 24, 48, 72, and 96 h indicate that the cellular response is not a uniform one. Failure of energy dispersive X-ray spectrophotometric analysis to demonstrate any intracellular incorporation of these cations (except for a weak cytoplasmic Cu peak at the 8.0 KEV position) suggests that the toxic actions of these cations at EC_{50} concentrations are exerted at the level of the plasma membrane.

Recent reviews (Boudou and Ribeyre 1989a, 1989b), and studies (Taylor 1989; Taylor and Stadt 1990; Visviki and Rachlin 1991) have recognized that examining the effects of divalent cations in various combinations is more representative, of the actual environmental problems faced by organisms, than are single metal studies. This recognition results from the realization that environmental loadings of cations from anthropogenic sources rarely involve single cation contributions, and if they do, the introduced cation will interact with a host of chemicals native to the receiving system. Thus, organisms potentially impacted by these toxicants face a multiple rather than a single toxicant insult. Working with algal models representative of the base of the aquatic food chain, our laboratory team has evaluated, under a variety of conditions, several key cations for their influence on growth (Rachlin *et al.* 1983; Rai *et al.* 1990; Rachlin and Grosso 1991; Visviki and Rachlin 1991). Using population growth as an end-point, we now report the interactions of the cations cadmium, cobalt, and copper, on the green alga *Chlorella vulgaris*, in terms of their actions being either additive, antagonistic, or synergistic.

Materials and Methods

The alga, *Chlorella vulgaris* (UTEX 30) was obtained as pure isolates from the Starr Culture Collection of Algae, University of Texas at Austin, and was grown and maintained in chelator-free modified Bristol's medium (Bold 1949) at a pH of 6.5 in 125 ml Erlenmeyer flasks. These flask cultures, containing 50 ml of the Bristol's medium, were incubated in a Sherer-Gillett RI-24 LTP growth chamber illuminated with Sylvania cool white fluorescent lamps supplemented with a 25-W incandescent light bulb to provide red light. Stock and experimental cultures were maintained in log phase of growth by the removal of 20 ml of medium and cells every seven days and replacing this with an equal volume of fresh sterile medium. Illumination was maintained at 280 foot candles (3.08 Klux, 7.84 W M^{-2}). The day/night program within the chamber was constant at 16 : 8 h and the incubation temperature was maintained at 19 ± 1°C.

Test solutions consisted of the modified Bristol's medium (pH 6.5) as control, or solutions of this medium containing the 96-h EC_{50} concentrations of the cations Cd, Co, and Cu, in the following combinations: Cd + Cu, Cd + Co, Cu + Co, and Cd + Cu + Co. Test solutions were made in modified Bristol's medium using the chloride salts of the cations in appropriate concentrations so that results are reported as concentrations of the cations alone (Rosko and Rachlin 1977; Rachlin *et al.* 1983). The EC_{50} concentrations used for Cu and Cd were, respectively, 0.18 ppm (2.8 μM) and 0.10 ppm (0.89 μM) and had been previously determined by Rosko and Rachlin (1977). The 96-h EC_{50} value for Co was determined in the current study and found to be 0.56 ppm (9.5 μM). All control and test concentration trials were run in triplicate, following the procedures described in Rachlin and Grosso 1991, for a maximum 96-h exposure period, with enough replicates (24 per test concentration) so that triplicate cell counts could be made every 24 h. Cell counts were made with a brightline hemocytometer, on well-mixed cultures, and since the cell counts for each flask of a triplicate run were within 10% of each other, the data for each triplicate were pooled for the determination of percent growth and 95% confidence intervals (Rachlin and Farran 1974; Rachlin and Grosso 1991).

Cation interactions were evaluated using modifications of Colby's formula as reviewed by Rai *et al.* (1981), and applied by Visviki and Rachlin (1991). Colby's original formula is, E = XY/100 (where E is the expected population growth of the alga as a percent of control growth. X and Y are the percent of control growth of the alga after exposure to cations X and Y, respectively. Values of E greater or less than the percent of control growth, respectively, indicate synergistic and antagonistic cation interactions, and values of E equal to the percent of control growth indicate an additive interaction). This for-

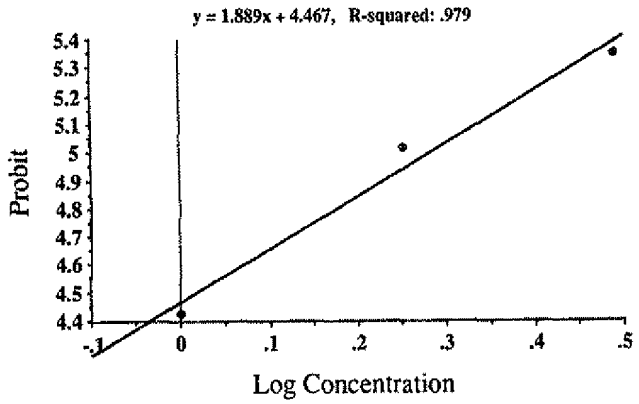

Fig. 1. Regression line of the probit response of *Chlorella vulgaris* to selected concentrations of cobalt

Table 1. Estimation of the EC_{50} values of cobalt from the percent response of *Chlorella vulgaris* after 96 h exposure, with respective regression equation

Conc (μM)	Conc (ppm)	Log conc	% Control growth	Empirical probit
5.4	0.32	−0.4949	63.8	5.3531
9.5	0.56	−0.2518	50.7	5.0175
17.0	1.00	0.0000	28.4	4.4290

$Y = 1.889X + 4.467$, $r^2 = 0.979$, Log $EC_{50} = -0.2821$, EC_{50} = 0.52 ± 0.038 ppm (9.0 μM), probability >95%. Confirmation run of 0.56 ppm (9.5 μM) Co yielded 50.7% reduction in growth

mula was modified in the following manner; if three variables are required, $E = (ABC)/100^2$, and if four variables are required, $E = (ABCD)/100^3$. Thus a general formula was developed, where $E = (V_1V_2...V_n)/100^{n-1}$ and n is the number of variables (V) being considered.

Cation incorporation into the exposed cells was evaluated for all test conditions by means of the X-ray energy dispersive approach, using a Hitachi H7000 EM equipped with a PGT System 4 Plus energy dispersive X-ray spectrometer in STEM mode at 75 Kv. This system was used on air dryed cation exposed and control cells following the procedures outlined in Baxter and Jensen (1980), Jensen *et al.* (1982), and Rai *et al.* (1990).

Results

The data for the regression line (Figure 1) and the regression equation of the probit response analysis from which the EC_{50} value for cobalt was determined is presented in Table 1. The estimated EC_{50} of 0.522 ± 0.38 ppm (9.0 μM) was confirmed in a series of runs in which test concentrations of cobalt at 0.56 ppm (9.5 μM) yielded a 50.7% reduction in growth of the test *Chlorella* cultures. Using this value, 9.5 μM for Co, and values of 2.8 μM and 0.89 μM, respectively representing the 96 h static trial EC_{50} concentrations of Cu and Cd for *Chlorella vulgaris*, a series of cation combination studies were performed. These results are presented in the "observed" column of Table 2. All metal combinations resulted in greater than 50% reduction in growth after 96-h of exposure when compared to control cultures. Assuming control growth to represent 100%, then combinations of Cd + Cu and Cu + Co resulted in respective growth reductions of 93.9% and 92.7%. The combinations of Cd + Co, and the three cation combinations (Cd + Cu + Co) resulted in essentially the same growth reduction (63.4% and 63.6%). Clearly, copper combined with either cadmium or cobalt is more toxic than the cadmium-cobalt combination. Surprisingly, the three cation combination is no more toxic than the cadmium-cobalt combination, and considerably less toxic than either the cadmium-copper or copper-cobalt combinations.

Table 2. Comparison between expected and observed percent of control growth of *Chlorella vulgaris* (± 95% confidence intervals) following 96 h exposure to metal combination

Metal combinations	Expected	Observed
Cd + Cu	25.0	6.1 ± 0.09
Cu + Co	25.0	7.3 ± 0.09
Cd + Co	25.0	36.6 ± 0.17
Cd + Cu + Co	12.5	36.4 ± 0.17

Table 3. Percent of control growth of *Chlorella vulgaris* (± 95% confidence intervals) at each 24 h interval of exposure to metal combinations

Time (h)	Cu + Co	Cd + Cu	Cd + Co	Cd + Cu + Co
24	12.8 ± 0.12	18.9 ± 0.14	44.0 ± 0.18	101.0 ± 0.04
48	31.5 ± 0.17	13.6 ± 0.12	58.7 ± 0.18	37.8 ± 0.17
72	14.2 ± 0.12	4.9 ± 0.08	39.9 ± 0.18	19.4 ± 0.14
96	7.3 ± 0.09	6.1 ± 0.09	36.6 ± 0.17	36.4 ± 0.17

To evaluate whether these cation combinations are interacting in an additive, synergistic, or antagonistic manner, a modification of Colby's formula was applied. Table 2 presents data in which the expected and observed growth responses are compared. It can be seen that the combinations of cadmium-copper, and copper-cobalt both produced greater reductions in growth than would be predicted by application of Colby's formula; and that both the cadmium-cobalt, and three cation combinations

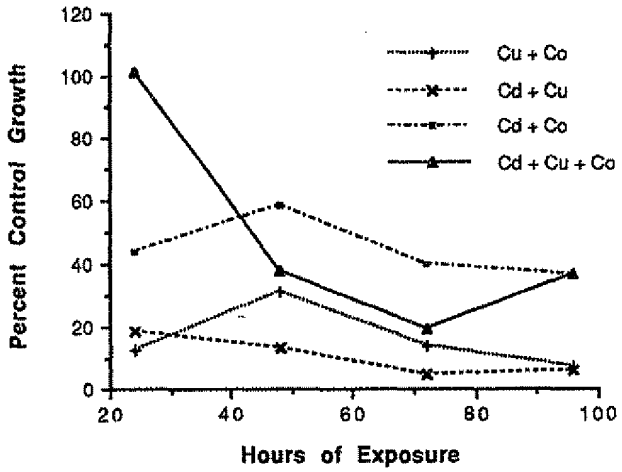

Fig. 2. Percent response of *Chlorella vulgaris* to exposure to various cation combinations

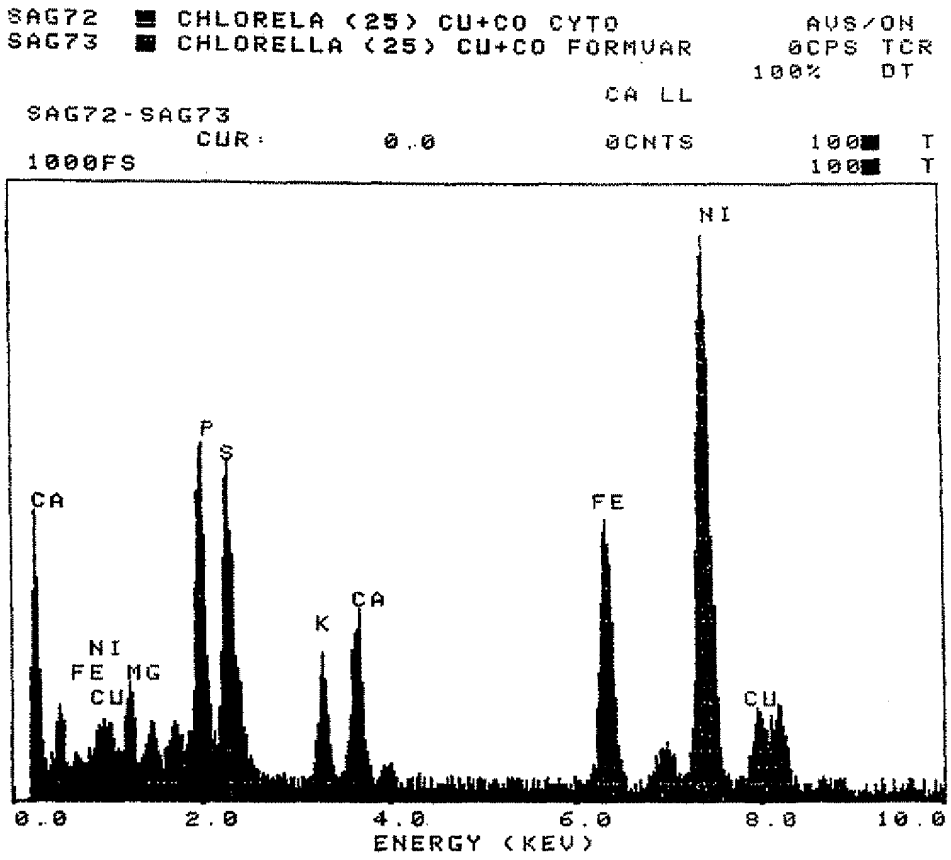

Fig. 3. Energy dispersive X-ray analysis spectra of *Chlorella vulgaris* to the cation combination of Cu + Co. Cytoplasm with formvar platform background subtracted

resulted in growth responses greater (lower percent reduction) than predicted by application of the formula.

In order to evaluate the growth response of *Chlorella vulgaris* to these metal combinations in time frames shorter than the standard 96-h static trial procedure, replicate cultures were set up so that counts could be made at 24 h intervals over the entire 96 h test procedure. In all the bimetal combinations (Table 3, Figure 2) growth of the cells was less than 50% of control values at 24 h. The curves for Cu + Co and Cd + Co were approximately parallel throughout the 96 h exposure, with both showing a slight recovery at 48 h and then a continued decline in growth to the final 96 h low values. The Cd + Cu curve does not show this 48 h recovery, but instead shows a continued decline in growth through 72 h and then leveled off for the remaining 24 h of the study. The tri-metal combination showed a different response; at 24 h growth was same as in control cultures, and then there was a dramatic decline in growth at 48 h (38% of control), and at 72 h (19.4% of control). At 96 h, there was a slight recovery to 36.4% of control, which is the same level achieved by the Cd + Co bimetallic combination at the end of the 96 h test run.

Energy dispersive X-ray spectrometric studies for evaluation of cellular incorporation of metals yielded negative results for all metal combination trials. That is there was no evidence of any intracellular metal incorporation. Only the Cu + Co combination indicated any metal incorporation (Figures 3, 4, and 5); these figures, respectively, show the Cu + Co spectra for (1) the cytoplasm with the formvar platform background subtracted, (2) the spectra of the polyphosphate body, and (3) the spectra of the polyphosphate body with the background cytoplasm subtracted. A weak cytoplasmic copper peak, indicating slight cellular incorporation, is clearly evident (Figures 3, 4) at the 8.0 KEV position.

Discussion

The toxicity of cobalt to *Chlorella vulgaris* (9.0 μM) is less than either the toxicity of Cu (2.8 μM) or Cd (0.89 μM). The series for toxicity of these three cations to *Chlorella vulgaris* is Cd > Cu > Co. In confirmation runs a concentration of 9.5 μM cobalt yielded 50.7% reduction in the population growth of the alga after 96 h of exposure (Table 1). This was the cobalt dose selected for use in the combined metal studies, as it represents an actual tested EC_{50} concentration rather than the value of 9.0 μM estimated from the regression response curve (Figure 1).

All metal combinations resulted, after 96 h of exposure, in greater than the 50% growth reduction of the single cation treatments. Using our modification of Colby's formula as the response predictor, combinations of Cd + Cu and Cu + Co resulted in reductions in growth greater than that expected for these bimetallic combinations, and are, therefore, acting in a synergistic fashion (Table 2). This contrasts with the bimetallic combination of Cd + Co, and the tri-metal combination (Cd + Cu + Co), which gave growth reductions less than ex-

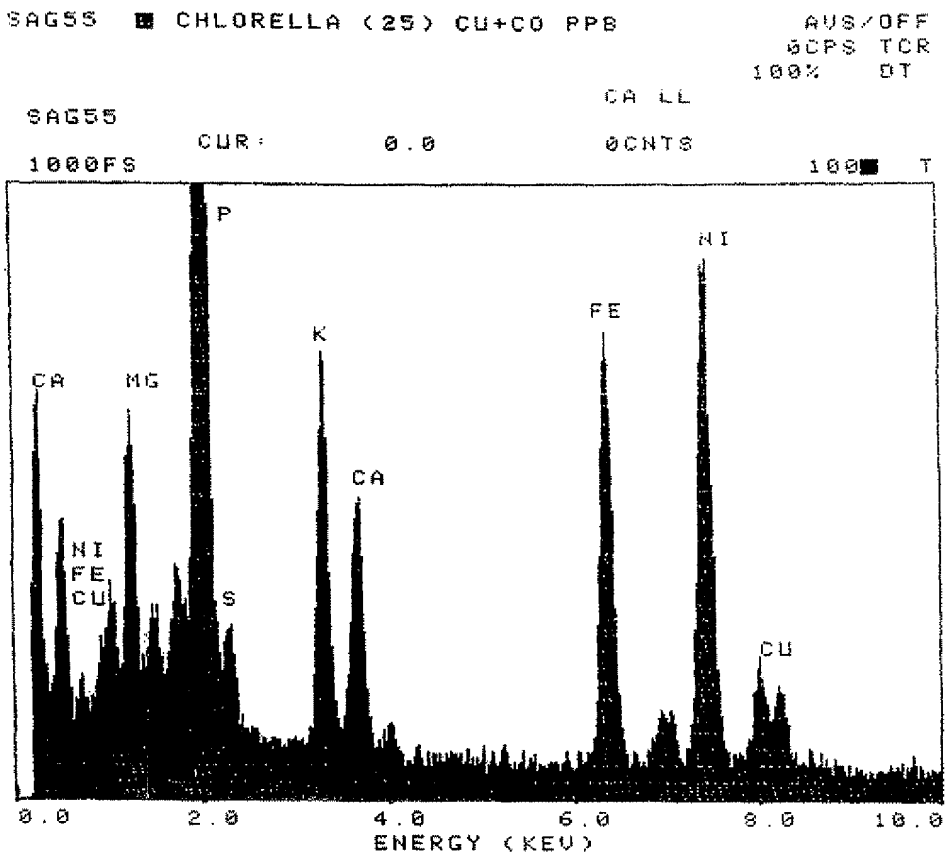

Fig. 4. Energy dispersive X-ray analysis spectra of *Chlorella vulgaris* to the cation combination of Cu + Co. Polyphosphate body

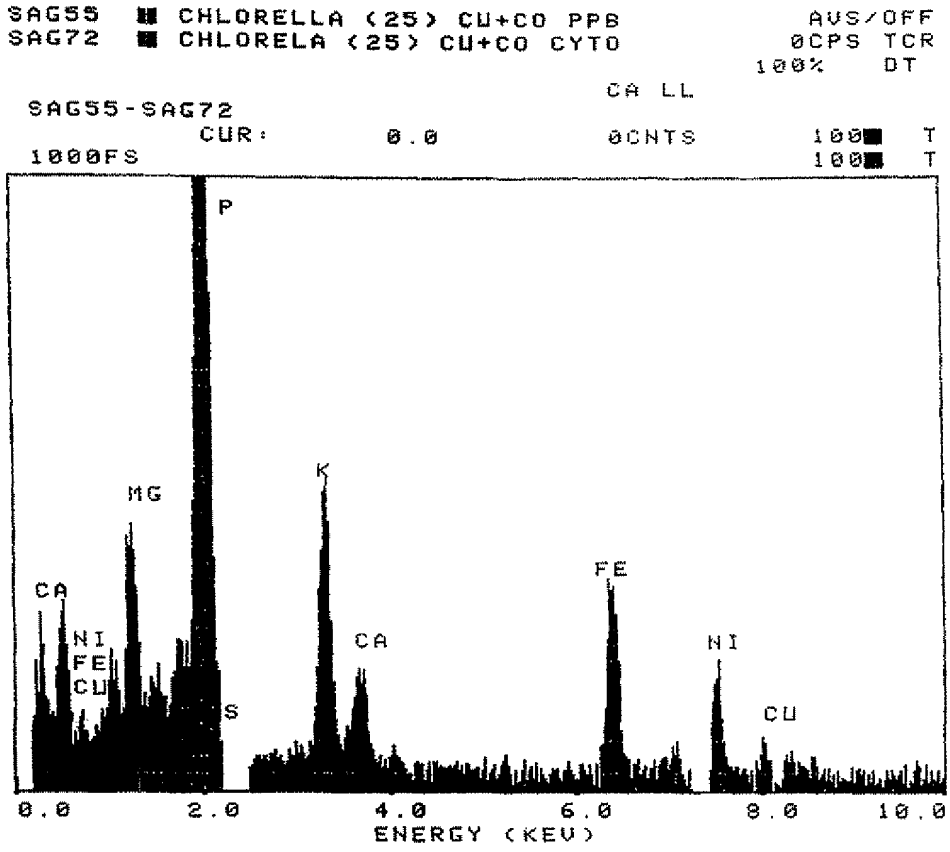

Fig. 5. Energy dispersive X-ray analysis spectra of *Chlorella vulgaris* to the cation combination of Cu + Co. Polyphosphate body with background cytoplasm subtracted

pected for these combinations, indicating antagonistic interactions. These data demonstrate that Cu combined with either Cd or Co is more toxic than either Cd combined with Co or the tri-metal combination. The combination of the most toxic cation (Cd) with the least toxic action (Co) resulted in antagonistic interactions, even when Cu was added. The antagonistic interaction of Cd and Co seems to be great enough to overcome the demonstrated synergism between Cd and Cu. These results, coupled with the results of the energy dispersive X-ray spectrometric studies, in which no intracellular metal incorporation, other than a weak cytoplasmic copper peak at the 8.0 KEV position, was found (Figure 3, 4, and 5) strongly suggest that the effects of these metal interactions result from membrane phenomena rather than intracellular toxicity. That metals compete for adsorption sites on the plasma membrane and evoke a toxicity response by affecting what traverses the membrane is well known (Rosko and Rachlin 1975, 1977; Rai *et al.* 1981; Rachlin *et al.* 1982, 1984, 1985; Sunda 1988/89; Majidi *et al.* 1990; Xue and Sigg 1990; Ionouhe *et al.* 1991; Visviki and Rachlin 1991). The non-linear response to these cation combinations, when examined at each 24 h time interval (Table 3, Figure 2), indicates that the toxic response is not uniform over time. This is consistent with a response resulting from alterations in membrane permeability. That cations interact with sulfhydryl groups on proteinaceous membranes to produce -S-metal-S-bridges, which can then alter membrane permeability was demonstrated by Rothstein (1959), and Simkiss (1979) indicated the importance of proteins and lipoproteins in trapping metals, which provides a mechanism for removing cations from the cell as a detoxification mechanism. These actions might account for the slight recovery (Figure 2) observed at 48 h in the Cd + Co and Cu + Co trials and in the tri-metal (Cd + Cu + Co) combination at 96 h.

Acknowledgments. This research was supported in part by National Institute of General Medical Sciences Grant # 2SO6 GMO8 225/07, Subproject No. 443080 to J.W. Rachlin.

References

Baxter M, Jensen TE (1980) A study of methods for *in situ* X-ray energy dispersive analysis of polyphosphate bodies in *Plectonema boryanum*. Arch Microbiol 126:213–215

Bold HC (1949) Morphology of *Clamydomonas chlamydogama* sp. nov. Bull Torr Bot Club 76:101–108

Boudou A, Ribeyre F (1989a) Aquatic ecotoxicology: Fundamental concepts and methodologies. Vol. I CRC Press Inc, Boca Raton, FL

———, ——— (1989b) Aquatic ecotoxicology: Fundamental concepts and methodologies. Vol. II. CRC Press Inc, Boca Raton, FL

Ionouhe M, Inagawa A, Morita M, Tohoyama H, Joho M, Murayama T (1991) Contributions of cell wall and metal-binding peptide to Cd and Cu tolerances in suspension-culture cells of tomato. Bot Mag Tokyo 104:217–482

Jensen TE, Rachlin JW, Jani V, Warkentine B (1982) An X-ray energy dispersive study of cellular compartmentalization of lead and zinc in *Chlorella saccharophila* (Chlorophyta), *Navicula incerta* and *Nitzschia closterium* (Bacillariophyta). Environ Exp Bot 22:319–328

Majidi V, Laude DA, Holcombe JA (1990) Investigations of the metal-algae binding site with ^{113}Cd nuclear magnetic resonance. Environ Science Technol 24:1309–1312

Rachlin JW, Farran M (1974) Growth response of the green algae *Chlorella vulgaris* to selected concentrations of zinc. Water Res 8:575–577

Rachlin JW, Grosso A (1991) The effects of pH on the growth of *Chlorella vulgaris* and its interactions with cadmium toxicity. Arch Environ Contam Toxicol 20:505–508

Rachlin JW, Jensen TE, Baxter M, Jani V (1982) Utilization of morphometric analysis in evaluating response of *Plectonema boryanum* (Cyanophyceae) to exposure to eight heavy metals. Arch Environ Contam Toxicol 11:323–333

Rachlin JW, Jensen TE, Warkentine B (1983) The growth response of the diatom *Navicula incerta* to selected concentrations of the metals: cadmium, copper, lead and zinc. Bull Torr Bot Club 110:217–223

———, ———, ——— (1984) The toxicological response of the alga *Anabaena flos-aquae* (Cyanophyceae) to cadmium. Arch Environ Contam Toxicol 13:143–151

———, ———, ——— (1985) Morphometric analysis of the response of *Anabaena flos-aquae* and *Anabaena variabilis* (Cyanophyceae) to selected concentrations of zinc. Arch Environ Contam Toxicol 14:395–402

Rai LC, Gaur JP, Kumar HD (1981) Phycology and heavy metal pollution. Biol Rev 56:99–151

Rai LC, Jensen TE, Rachlin JW (1990) A morphometric and X-ray energy dispersive approach to monitoring pH-altered cadmium toxicity in *Anabaena flos-aquae*. Arch Environ Contam Toxicol 19:479–487

Rosko JJ, Rachlin JW (1975) The effect of copper, zinc, cobalt and manganese on the growth of the marine diatom *Nitzschia closterium*. Bull Torr Bot Club 102:100–106

———, ——— (1977) The effect of cadmium, copper, mercury, zinc and lead on cell division, growth, and chlorophyll a content of the chlorophyte *Chlorella vulgaris*. Bull Torr Bot Club 104:226–233

Rothstein A (1959) Cell membrane as site of action of heavy metals. Fed Proc Fed Amer Soc Exp Biol 18:1026–1028

Simkiss K (1979) Metal ions in cells. Endeavour 3:2–6

Sunda WG (1988/89) Trace metal interactions with marine phytoplankton. Biol Oceanog 6:411–442

Taylor GJ (1989) Multiple metal stress in *Triticum aestivum*. Differentiation between additive, multiplicative, antagonistic, and synergistic effects. Can J Bot 67:2272–2276

Taylor GJ, Stadt KJ (1990) Interactive effects of cadmium, copper, manganese, nickel, and zinc on root growth of wheat (*Triticum aestivum*) in solution. In: Van Beusichem ML (ed) Plant nutrition—physiology and applications. Proceedings of the 11th international plant nutrition colloquium, Wageningen, The Netherlands, July 30 to August 4, 1989. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 317–322

Visviki I, Rachlin JW (1991) The toxic action and interactions of copper and cadmium to the marine alga *Dunaliella minuta*, in both acute and chronic exposure. Arch Environ Contam Toxicol 20:271–275

Xue HB, Sigg L (1990) Binding of Cu(II) to algae in a metal buffer. Water Res 24:1129–1136

Manuscript received June 4, 1992 and in revised form July 3, 1992.

EXHIBIT D

Marine Biology 59, 193–200 (1980)

MARINE BIOLOGY

Combined Effect of the Chlorides of Mercury and Copper in Sea Water on the Euryhaline Amphipod *Gammarus duebeni*

Shelagh M. Moulder*

Department of Oceanography, The University; Southampton, SO9 5NH, England

Abstract

The possible interactive effect of the chlorides of copper and mercury on the euryhaline amphipod *Gammarus duebeni* in 100% sea water was examined using the following indices: (i) 96 h LC50 values, (ii) urine production rates and (iii) degree of mercury accumulation. Both (a) the interaction of the chlorides of mercury and copper together in solution and (b) the influence of cupric chloride pre-treatment of individuals prior to exposure to mercuric chloride were investigated. Presence of a sublethal level of cupric chloride protected *G. duebeni* against the toxic action of mercuric chloride. Cupric chloride pretreatment was not so effective. The nature of the interaction between mercury and copper is discussed.

Introduction

Relatively little work has been undertaken to investigate the effect on aquatic organisms of exposure to 2 metals together. However, in the late 1940's the toxicity of the salts of mercury and copper were examined both alone and when mixed, since these metals were commonly used in anti-fouling compounds at that time (Barnes and Stanbury, 1948; Pyefinch and Mott, 1948; Hunter, 1949).

Barnes and Stanbury (1948) demonstrated that mixtures of cupric sulphate and mercuric chloride were far more toxic to the copepod *Nitocra spinipes* than either of the salts separately and, furthermore, were even more toxic than the simple addition of the toxicities of both metals concerned. In a later investigation of bipartite mixtures of poisons Corner and Sparrow (1956) examined the toxicity of copper and mercury to *Artemia salina* and *Acartia clausi*. Their results revealed that more-than-additive effects were only obtained with mixtures of copper and mercury salts at restricted metal concentrations, and that the size of the synergistic effect produced was dependent upon the species of organism being tested. Corner and Sparrow also found that varying the mercury compound used in the experiments produced a different effect on the organisms.

More recently, Murakami *et al.* (1976) reported only additive effects on developing sea urchin eggs when mercury and copper were combined in filtered sea water as the chloride salts of the metals, and Reeve *et al.* (1977) using copepod populations showed that mercury produced lethal effects at concentrations some three times lower than those of copper but that a mixture of 2.5 $\mu g\, l^{-1}$ copper and 2.5 $\mu g\, l^{-1}$ mercury acted essentially the same as 5 $\mu g\, l^{-1}$ mercury alone.

The interaction of mercury and copper is complex, with widely varying results being obtained with different animal species, mercury and copper compounds and equitoxic combinations.

In the present work *Gammarus duebeni* was used to attempt to further elucidate the nature of the effect of the mercury–copper interaction on living organisms. The effect of mercury in combination with copper on mercury toxicity, urine production rate and mercury accumulation was studied.

Materials and Methods

Collection of Animals

Individuals were collected weekly by hand net from the salt marsh at Totton, which lies at the junction of the River Test and the head of Southampton Water, England, and kept until use in 100% department aquarium sea water; salinity 33 ± 2 ‰S. The water temperature was maintained at 15° ± 1 C° and the water was aerated until the experiments commenced. During this period the *Gammarus duebeni* were fed with detrital material,

*Present address: 7, Summerfield Road, Holy Cross, Clent, Stourbridge, West Midlands, DY9 9RG, England

0025-3162/80/0059/0193/$01.60

brought in with the individuals at the time of collection, or with *Enteromorpha* spp. but were not fed for 48 h prior to or during the experiments. In all experiments the *G. duebeni* were chosen at random from healthy individuals acclimatised to the experimental conditions of light, temperature and salinity for at least 3 d and no more than 10 d.

Preparation of Stock Metal Solutions

Mercuric chloride was made up as required using the anhydrous salt (Molecular Wt. 271.5) in 0.1N HCl to prevent precipitation of mercuric carbonate. Cupric chloride solution was prepared in glass-distilled water using the dihydrate (Molecular Wt. 170.5). Both compounds were supplied as the analar salt by BDH. The concentration of the stock solutions was such that 1 ml of stock solution diluted to 1 l gave the required metal concentration for experimental use. Metal concentrations are stated in $\mu g\ l^{-1}$ or $\mu g\ ml^{-1}$ with respect to the metal cations in the compounds. Stock solutions were made weekly.

Toxicity Testing

Ninety-six-hour static tests were performed at 15 °C in a constant temperature room with a 12 hour dark:12 hour light (12 h D:12 h L) regime. Glass dishes were used which had been acid-washed in 25% HCl and rinsed thoroughly in distilled water. Forty adult male *Gammarus duebeni*, in the weight range 60 to 90 mg, were used in each test metal concentration and also in a control solution with no added metal. Each individual was allowed 50 ml of solution made up from 100% department aquarium sea water and the media were changed every 24 h. The pH of all solutions was 7.5 ± 0.5.

Urine Production

Chromium-51-labelled ethylene diamine tetraacetic acid injection fluid obtained from the Radiochemical Centre, Amersham, U.K., was used in this work to measure urine production. Each millilitre of ^{51}Cr-EDTA injection, after concentration by freeze drying, contained 6 to 10 mg ml^{-1} of chromium-EDTA (calculated on the basis of a 1:1 anhydrous complex of chromium ions with EDTA) and 19 to 34 mg ml^{-1} of sodium edetate BPC. The radioactive concentration was 0.9 to 1.1 mCi ml^{-1} of solution and the specific activity lay within the range of 700 to 1000 $\mu Ci\ mg^{-1}$ of Cr. The precise amounts varied with each batch of isotope.

Adult males (weight 70 to 90 mg) were injected between the mesosomal or metasomal plates with 1 to 2 μl of ^{51}Cr-EDTA. They were then returned to a large volume of clean sea water to allow recovery and the removal of any surface radioactive material not injected into the body. After 6 h recovery those individuals which appeared normal were counted for tracer activity to ascertain whether injection had been successful, and were then transferred to the test media. Each individual was kept in a 100 ml beaker containing 50 ml of solution and the beakers were placed in a water bath at 15 ° ± 1 C° with a 12 h D:12 h L regime. The media were changed every 24 h.

Using a well-type solid crystal scintillation counter, each individual was counted for tracer activity twice a day for 3 d following injection. Results obtained from any gammarids which moulted or died before the end of the experiment were discounted. Corrections were made for background radiation and isotope decay to the whole-body ^{51}Cr tracer counts obtained. Rates of urine production determined under various experimental conditions were compared by using the $t½$ values (time for 50% of the injected isotope to be cleared from the body). The significance of the results was calculated using Student's *t*-test.

Mercury Accumulation

Experimental media of the required concentrations were made up from stock mercuric chloride solutions and were spiked with ^{203}Hg of high specific activity (0.3 to 2.0 mCi Hg^{-1}) to give a gamma count of approximately 20 to 25 cs^{-1} above background. After weighing, adult male *Gammarus duebeni* were placed in glass dishes containing the spiked mercuric chloride solutions allowing 50 ml of solution per individual and 10 individuals for each replicate experiment. All tests were conducted in 100% sea water under the conditions of the urine production experiments. At specific intervals of time the *G. duebeni* were removed from the uptake media, rinsed briefly in media containing no mercury contamination and counted for tracer activity using the well-type solid crystal scintillation counter. At each sampling time two 1 ml aliquots of medium from each experimental dish were also counted.

Results

Toxicity testing

The possible interactive effect of the toxicity of mercury and copper was investigated in two ways; firstly, the toxicity of copper and mercury together in solution, and secondly, the toxicity of mercury after pretreatment of the individuals with copper. In the control experiments the toxicity of mercury and copper alone was assessed. Mortality was monitored every 24 h over the 96 h test period, the criterion of death being total lack of movement of a specimen after repeated stimulation with forceps.

On being placed in toxic solutions the gammarids underwent an initial period of increased activity, most probably due to handling and avoidance reactions. Fol-

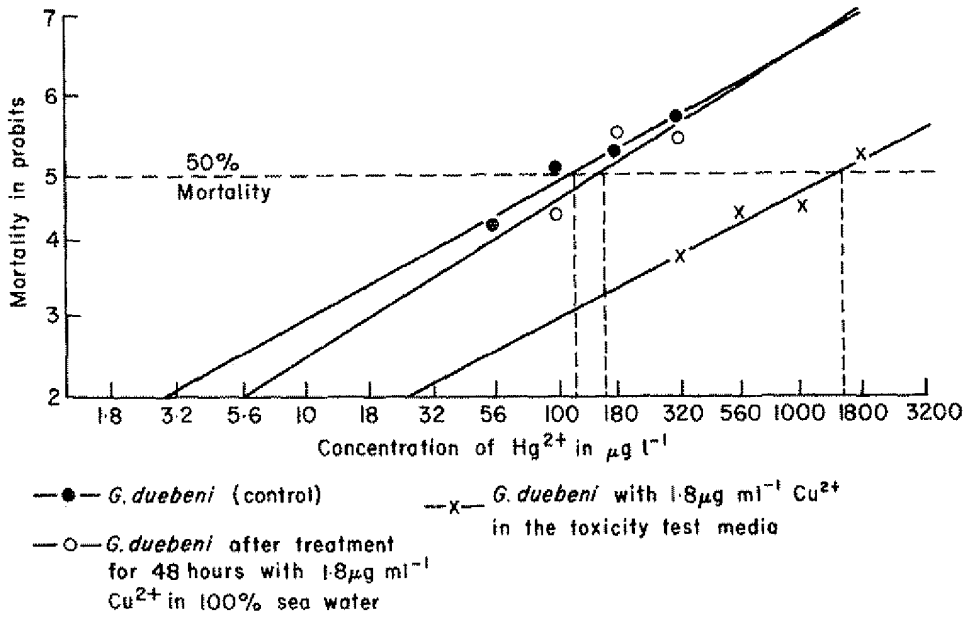

Fig. 1. *Gammarus duebeni.* Toxicity of inorganic mercury as mercuric chloride in 100% sea water, with and without the pretreatment of, or presence of copper as cupric chloride

Table 1. *Gammarus duebeni.* Ninety-six-hour LC50 and threshold values in 100% sea water with inorganic mercury (as mercuric chloride) after pretreatment with 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ for 48 h (as cupric chloride), or with 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ (as cupric chloride) present in the toxicity test media. (Values as $\mu g\ l^{-1}$). Dash = not determined

Test conditions	LC50	Threshold
Cu^{2+} only	>10,000	–
Hg^{2+} only	122	3
Hg^{2+} after pretreatment of the test individuals with 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$	165	5.6
Both Hg^{2+} and 1.8 $\mu g\ ml^{-1}$ Cu^{2+} in the test media	1520	26

lowing this activity, swimming gradually decreased and towards the end of the survival time ceased altogether, although the pleopods continued to beat irregularly. Finally death occurred. Mortality also occasionally occurred in the control group. This was almost entirely due to moulting individuals being eaten by others, and it gives an indication of the 'natural' mortality taking place over the experimental period. Although moulting occurred in the toxic solutions the gammarids were rarely eaten due to the sluggish and often moribund condition of the metal-affected individuals. Correction for this 'natural' mortality was made by the simple method of Tattersfield and Morris (1924).

The results are shown in Fig. 1. The lines of best fit were drawn after linear regression analysis.

Ninety-six-hour LC50 values were taken from the graph. Probit 5 is equivalent to 50% mortality. An estimate of the maximum concentrations of the metals having no toxic effect under the experimental conditions was also made from the graph. These are termed the threshold concentrations (Table 1). Probit 1.9098 is equivalent to 0.1% mortality. Metal concentrations below the threshold are sublethal in the 96 h period.

Toxicity tests conducted in 100% sea water revealed that pretreatment of *Gammarus duebeni* with 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ as cupric chloride for 48 h had no significant effect on the toxicity of mercury to the gammarid, but that the presence of a small amount of copper in the media did significantly reduce toxicity. In fact, with 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ added to the media, the 96 h LC50 value for mercury was approximately ten times greater than that of the control (Table 1). A higher LC50 value is indicative of increased tolerance. Thus, only combination of the chlorides of both mercury and copper together in sea water resulted in less than additive effects on *G. duebeni*. It would appear that under these conditions copper is in some way protecting the gammarids from mercury poisoning. There are 3 possible reasons for this: (i) copper may occupy binding sites on the surface of the gammarid which would otherwise be occupied by mercury, thus reducing mercury uptake and accumulation; (ii) a copper–mercury complex might be formed in sea water reducing mercury availability to the gammarid; (iii) copper may in some way detoxify mercury within the tissue. These are fully discussed later.

Urine Production

A change in the urine production rate as an indication of environmental stress in both invertebrates and vertebrates is well known and has been used to assess sublethal pollutant effects (Lloyd and Orr, 1969; Maddrell and

Table 2. *Gammarus duebeni.* ^{51}Cr -EDTA clearance in 100% sea water–exposure to two metals together

Test medium	Mean $t½$ value (hours)	Standard error	No. of individuals (N)	Student's t value	Significance P
100% sea water (control)	156.5	± 28.9	26	–	–
18 $\mu g\ l^{-1}$ Hg^{2+} alone	76.9	± 12.1	17	5.082	< 0.0005
1.8 $\mu g\ ml^{-1}$ Cu^{2+} alone	81.9	± 16.5	7	3.459	< 0.0025
18 $\mu g\ l^{-1}$ Hg^{2+} and 1.8 $\mu g\ ml^{-1}$ Cu^{2+} together	81.3	± 23.9	9	3.400	< 0.0025

Casida, 1971; Lockwood and Inman, 1975; Lloyd and Swift, 1976).

Using *Gammarus duebeni* changes in the rate of urine production were apparent with the following experimental media: (i) 100% sea water (control); (ii) 18 $\mu g\ l^{-1}$ Hg^{2+} in 100% sea water; (iii) 1,800 $\mu g\ l^{-1}$ Cu^{2+} in 100% sea water; and (iv) 18 $\mu g\ l^{-1}$ Hg^{2+} and 1,800 $\mu g\ l^{-1}$ Cu^{2+} in 100% sea water.

The $t½$ values for 18 $\mu g\ l^{-1}$ Hg^{2+} and 1.8 $\mu g\ ml^{-1}$ Cu^{2+} together in the test media, and for 18 $\mu g\ l^{-1}$ Hg^{2+} or 1.8 $\mu g\ ml^{-1}$ Cu^{2+} separately, show that there is a significant ($P < 0.0025$) diuresis in each case compared with the control (Table 2). However, from a comparison of the results it is evident that there is no significant difference in the extent of the diuresis caused by mercury and copper together and that due to mercury or copper alone. (Using Student's t-test on the results for copper alone against mercury and copper together, a value for t of 0.023 ($P = 0.495$ to 0.49) is obtained, and for mercury alone as against mercury and copper together t is found to equal 0.497 ($P = 0.35$ to 0.30).

Thus a mixture of mercury (as mercuric chloride) and copper (as cupric chloride) together in the test medium resulted in less than additive effects when their influence was assessed by measuring urine production in *Gammarus duebeni*.

Mercury Accumulation

The experimental conditions were as follows:

(i) a) Ten gammarids in 100% department aquarium sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ after pretreatment for 48 h in department aquarium sea water with no added mercury (control).
b) Ten gammarids in 100% department aquarium sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ after pretreatment for 48 h to 1.8 $\mu g\ ml^{-1}$ Cu^{2+} in 100% department aquarium sea water.
(ii) a) Ten gammarids in 100% department aquarium sea water containing 18 $\mu g\ l^{-1}$ Hg^{2+} (control).
b) Ten gammarids in 100% department aquarium sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ and 1.8 $\mu g\ ml^{-1}$ Cu^{2+}.
(iii) a) Ten gammarids in artificial sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ (control).
b) Ten gammarids in artificial sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ and 1.8 $\mu g\ ml^{-1}$ Cu^{2+}.

The recipe of Kester *et al.* (1967), slightly modified (Moulder, 1979), was used to prepare the artificial sea water.

(iv) a) Ten gammarids in photo-oxidised 100% department sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ (control).
b) Ten gammarids in photo-oxidised 100% department sea water containing 18 $\mu g\ l^{-1}$ $^{203}Hg^{2+}$ and 1.8 $\mu g\ ml^{-1}$ Cu^{2+}.

Prior to photo-oxidation the sea water was vacuum filtered through a Whatman glass microfibre filter. Approximately 0.25 ml of 50% hydrogen peroxide was added to each 100 ml aliquot of sea water before oxidation to act as a free radical source to facilitate the decomposition of organic material (Pineda, 1973). The sea water was irradiated in quartz silica tubes for 6 h with a 1,000 W U.V. lamp.

The experimental procedure and concentration of metals used in both of the organic free accumulation studies (Experiments iii and iv) was exactly the same as in Experiment ii except for the use of photo-oxidised or artificial sea water in the place of department aquarium water.

The results were plotted in terms of the concentration factor (CF) against time and are shown in Figs. 2 to 5. The vertical bars represent the standard error of the mean of the results. The term CF defines to what greater extent an aquatic organism accumulates a metal than its concentration in the surrounding water. Thus,

$$CF = \frac{\text{concentration in the organism}}{\text{concentration in water}}$$ (In comparable units e.g. per gm).

Interpretation of the results is based on the assumption that there is no isotopic effect; i.e. it is assumed

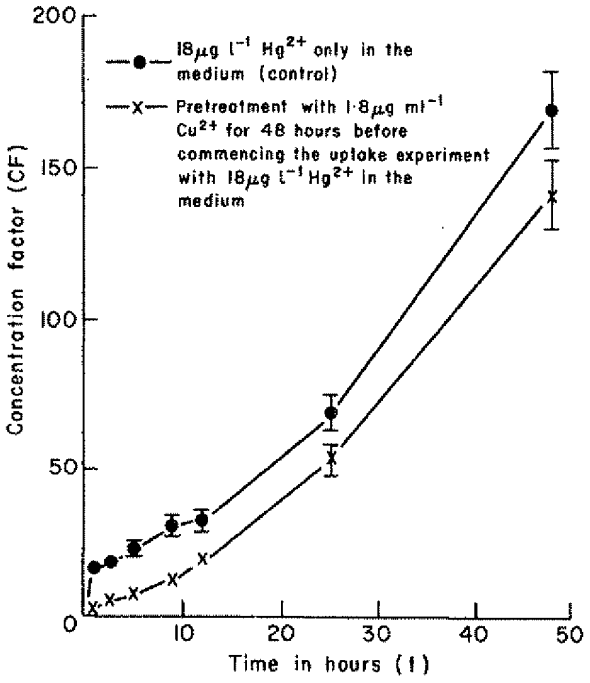

Fig. 2. *Gammarus duebeni*. Effect of copper on mercury accumulation in 100% sea water. I. Pretreatment with copper

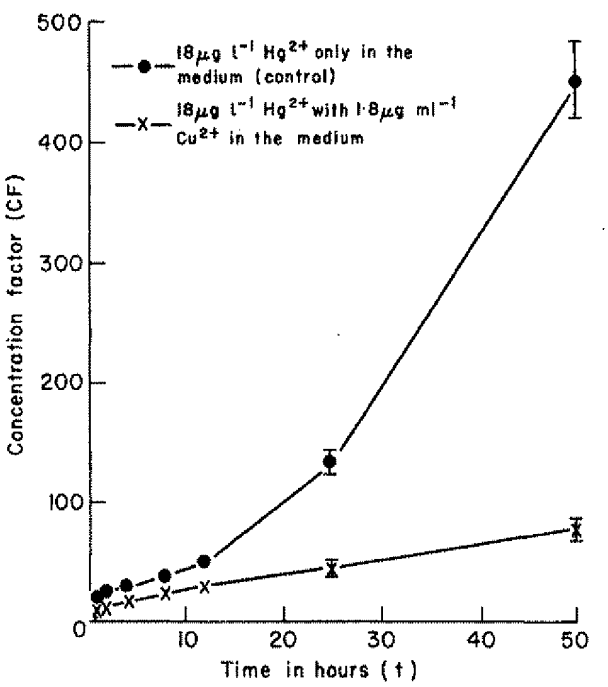

Fig. 4. *Gammarus duebeni*. Effect of copper on mercury accumulation in artificial sea water

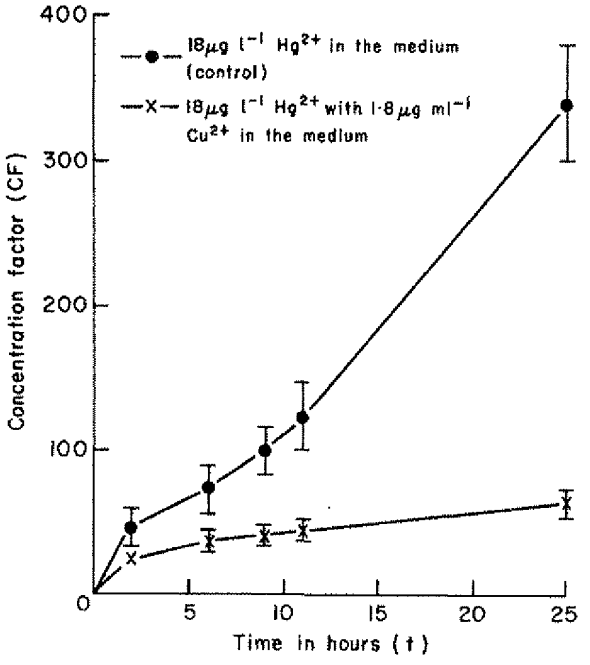

Fig. 3. *Gammarus duebeni*. Effect of copper on mercury accumulation in 100% sea water. II. Presence of copper in the uptake medium

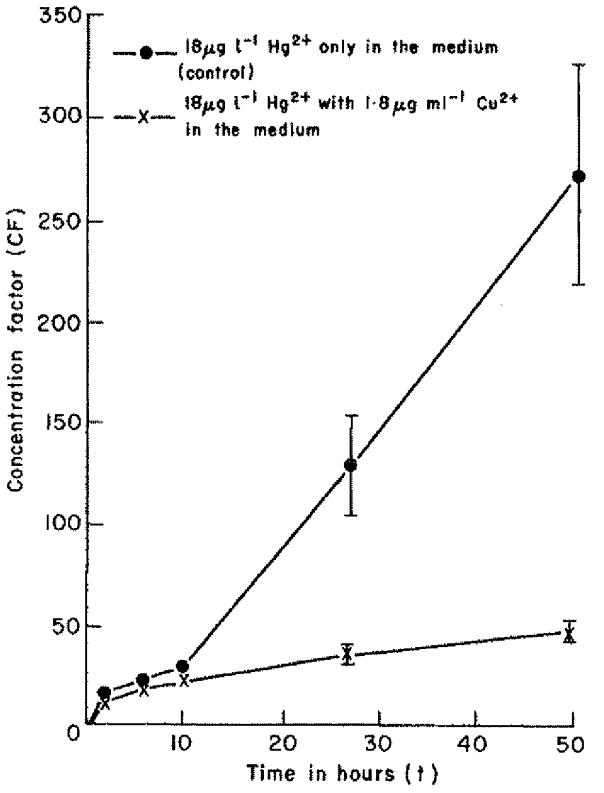

Fig. 5. *Gammarus duebeni*. Effect of copper on mercury accumulation in photo-oxidised 100% sea water

that the stable isotope of mercury and the radioactive isotope mercury-203 in the form of mercuric chloride are in the same physio-chemical form in sea water and have the same patterns of uptake, accumulation and loss.

During the period over which the experiments were conducted a certain degree of variability was noted in the CFs obtained for the control individuals in different experiments. Previous work on *Gammarus duebeni* had demonstrated the importance of size on mercury accumulation and therefore within any single experiment care was taken to use adult males within a narrow weight range and from the same population. It was possible for each experiment to form groups of 10 control and 10 experimental individuals with approximately the same mean wet weights. However, because of seasonal changes in size it was impossible to use specimens of the same weight for all the experiments. Other parameters such as time of year, stage in the moult cycle and genetic factors may also be important in causing the variability noted.

Use of Student's *t*-test reveals that there is a significant reduction (95% level or higher) in the CFs obtained for gammarids which had been pretreated with the sublethal level of copper compared with individuals which had no copper pretreatment (Fig. 2). This significant reduction in mercury accumulation is also obtained when gammarids are exposed to mercury and copper together. However, the presence of copper in the uptake medium results in a far more pronounced reduction in mercury accumulation (Fig. 3) compared with a control than that obtained by pretreatment of individuals with the same concentration of copper.

In both the organic-free media, a similar significant drop in mercury accumulation in the presence of a sublethal concentration of copper is still evident (Figs. 4 and 5). Thus it appears that the ability of copper to produce a reduction in the accumulation of mercury in sea water by *Gammarus duebeni* is not dependent upon the existence of organic matter in the medium.

Discussion

In the present work *Gammarus duebeni* was used to elucidate further the nature of the interaction between mercury and copper.

A reduction in the toxicity of inorganic mercury to *Gammarus duebeni* occurred with the presence of a sublethal amount of copper in the toxicity test medium. For example, the presence of 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ in the toxicity test medium increased the 96 h LC50 value for mercury as mercuric chloride from 122 $\mu g\ l^{-1}\ Hg^{2+}$ to 1520 $\mu g\ l^{-1} Hg^{2+}$, a more than ten-fold increase in inorganic mercury tolerance. However, pretreatment with 1.8 $\mu g\ ml^{-1} Cu^{2+}$ for 48 h immediately before the commencement of the toxicity test only increased the 96 h LC50 values to 165 $\mu g\ l^{-1} Hg^{2+}$. This value is not significantly different from that of the control (122 $\mu g\ l^{-1} Hg^{2+}$) of individuals untreated with copper either before or during the toxicity test. It would therefore appear that mercury and copper must be present at the same time for there to be a reduction in mercury toxicity (Fig. 1).

Corner and Sparrow (1956) revealed that preliminary treatment with a sublethal dose of copper markedly lowered the resistance of *Artemia* spp. to mercuric chloride and, to a lesser extent, mercuric iodide and and ethylmercuric chloride. This was not found to be the case with *Gammarus duebeni* here.

The protective effect of copper on inorganic mercury poisoning in *Gammarus duebeni* is again evident in the studies on mercury uptake and accumulation. Fig. 3 shows that the CF for mercury-203-labelled mercuric chloride from 18 $\mu g\ l^{-1}\ Hg^{2+}$ in 100% sea water after 25 h is 340 with no copper present in the uptake medium, and only 62 when 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ is available (a more than five-fold reduction in the CF). However, it can be seen that the copper and mercury must be together in the medium in order for mercury accumulation to be reduced so dramatically, as *G. duebeni* pretreated with 1.8 $\mu g\ ml^{-1}\ Cu^{2+}$ in 100% sea water prior to the commencement of the uptake experiment exhibited only a small reduction in CF compared with that obtained for individuals with no copper pretreatment (Fig. 2). After 48 h the CF for mercury as mercury-203-labelled mercuric chloride in *G. duebeni* from exposure to 18 $\mu g\ l^{-1}\ Hg^{2+}$ in 100% sea water is 169 with no copper pretreatment and 142 after 48 h of copper pretreatment (a statistically insignificant reduction).

Although there is no known formation in sea water of a complex containing copper and mercury ions in combination with organic matter, both copper and mercury separately have been shown to have an affinity for particulate and/or dissolved organics (Williams, 1969; Foster and Morris, 1971; Millward and Burton, 1975; Batley and Florence, 1976). The possibility that a complex was formed, containing mercury, copper and organic matter, in sea water, thus reducing mercury availability to the organism, was examined.

Uptake experiments performed in artificial sea water containing no organics and in 100% sea water filtered and irradiated with U.V. light to remove both particulate and dissolved organics revealed that the presence of 1.8 $\mu g\ ml^{-1} Cu^{2+}$ in the uptake medium significantly reduced mercury accumulation in *Gammarus duebeni* by a similar amount to that in ordinary 100% sea water (Figs. 3, 4 and 5). Hence, the presence of organic matter is not necessary for copper to act in a protective fashion towards poisoning by mercury of *G. duebeni*. It can therefore be assumed that a complex of mercury and copper ions with organic matter is either not produced or, if formed, is not influencing metal toxicity to the gammarid.

Complex formation from mercury and copper ions alone, without the involvement of organic matter, might occur and thus reduce metal availability to the organism, but this seems unlikely as there is no chemical reason why these 2 metal cations should form an associa-

tion. It must therefore be assumed that the protective action of copper on mercury poisoning of *Gammarus duebeni* does not occur in the aquatic medium *per se* but rather at the surface of the gammarid or inside the cells.

Both mercury and copper ions are known to bind to sulphydryl (SH) groups. The toxicity data (Table 1, Fig. 1) indicate the enormous tolerance that *Gammarus duebeni* shows to copper compared with that to inorganic mercury. It is proposed that the gammarid may be able to 'use' this tolerance to copper by selectively blocking the binding sites on the surface of the gammarid with copper in preference to mercury when the 2 metals are presented together in solution. However, there is no evidence to support this suggestion.

One attempt to establish the existence of competition for binding sites was made using dihydroxy dinaphthyl disulphide (DDD), a histochemical stain. Using this stain the presence of SH groups (known to be suitable binding sites for both copper and mercury) was demonstrated on the external surface of *Gammarus duebeni*. Both untreated and mercury or copper treated specimens were sectioned, after carnoy fixation, using a Leitz microtome. After using haematoxylin and eosin, a general stain for cytoplasm, to reveal whether the sections were satisfactory, fresh sections were stained with DDD. These showed the presence of SH groups on the cuticle (stained red) and in various tissues including muscle. If mercury or copper occupied the SH groups, the cuticle of a mercury or copper treated specimen should strain less heavily than that of a metal-free one. However, by eye there was no apparent difference in the degree of staining between untreated and mercury- or copper-treated specimens. Either the treatment of the specimens in fixing, processing and staining removed any bound metal, again exposing the SH groups, or the number of SH groups blocked by the metals is small relative to the total number of SH groups, or the staining technique is simply not sensitive enough to detect a change. It was therefore not possible to determine whether copper or mercury bound to SH groups on the surface of *G. duebeni* or whether there was any interaction between these metals acting at the gammarid surface.

It seems improbable that copper is acting protectively within the tissue by a detoxification mechanism for two reasons. Firstly, mercury detoxification by copper would not lead to a reduction in the total amount of mercury accumulated but would alter the chemical form of the metal, after accumulation, to a less toxic species. Secondly, if a detoxification mechanism within tissues did occur it is probable that copper accumulated from pretreatment experiments would be just as effective a source as copper accumulated at the same time as mercury.

It was also noted in the studies of mercury-induced diuresis that the presence of a sublethal level of copper resulted in less-than-additive diuretic effects, i.e. again copper is protecting the gammarids from the toxic effect of mercury (Table 2).

Thus 3 independent criteria (toxicity testing, metal accumulation and metal induced diuresis) have shown that the presence of a sublethal level of copper in 100% sea water reduces the poisonous nature of inorganic mercury to *Gammarus duebeni*.

No attempt has been made in this investigation to determine what influence, if any, a sublethal concentration of mercury has on copper toxicity and accumulation. Such research might prove valuable. There is also a need for further examination of the effect of differing concentrations of mercury and copper in combination not only using *Gammarus duebeni* but also other marine species.

It appears to be quite probable that at certain concentrations mercury and copper act antagonistically and that at others they act additively or even synergistically. Such a change in the effect of combination of heavy metals with concentration has been shown in studies of the growth rate of a bacterivorous, sediment-living ciliate protozoan, *Cristigera* sp. (Gray, 1974).

In 2 or 3 factor interactions Gray found synergistic effects at high metal concentrations but antagonistic effects at low metal concentrations. The varying effect of the interaction between mercury and copper on *Artemia* spp. has been previously noted (Corner and Sparrow, 1956).

In any attempt to set water quality standards the interaction of various pollutants is of utmost importance. There are many different types of effluent released into the aquatic environment and a vast number of possible pollutant combinations, many of which have not been investigated or even considered. The possibility of synergistic interaction between 2 or more compounds should always be considered or it could lead to the overestimation of environmentally 'safe' levels.

Acknowledgements. This work was funded by the Natural Environment Research Council under the supervision of Dr. A. P. M. Lockwood.

Literature Cited

Barnes, H. and F. A. Stanbury: The toxic action of copper and mercury salts both separately and when mixed on the harpacticid copepod, *Nitocra spinipes* (Boeck) J. exp. Biol. *25*, 270–275 (1948)

Batley, G. E. and T. M. Florence: Determination of the chemical forms of dissolved cadmium, lead and copper in sea water. Mar. Chem. *4*, 347–363 (1976)

Corner, E. D. S. and B. W. Sparrow: The modes of action of toxic agents. I. Observations on the poisoning of certain crustaceans by copper and mercury. J. mar. biol. Ass. U.K. *35*, 531–548 (1956)

Foster, P. and A. W. Morris: The seasonal variation of dissolved ionic and organically associated copper in the Menai Straits. Deep Sea Research *18*, 231–236 (1971)

Gray, J. S.: Synergistic effects of three heavy metals on growth rates of a marine ciliate protozoan. *In*: Pollution and physiology of marine organisms, pp 465–483. Ed. by F. J. Vernberg and W. B. Vernberg. New York and London : Academic Press 1974

Hunter, W. R.: The poisoning of *Marinogammarus marinus* by cupric sulphate and mercuric chloride. J. exp. Biol. *26*, 113–124 (1949)

Kester, D. R., I. W. Duedeall, D. N. Connors and R. M. Pytkowick: Preparation of artificial sea water. Limnol. Oceanogr. *12*, 176–179 (1967)

Lloyd, R. and L. D. Orr: The diuretic response by rainbow trout to sub-lethal concentrations of ammonia. Water Research *3*, 335–344 (1969)

Lloyd, R. and D. J. Swift: Some physiological responses by fresh-water fish to low dissolved oxygen, high CO_2, NH_3 and phenol with particular reference to water balance. *In*: The effects of pollutants on aquatic organisms, p 47. Ed. by A. P. M. Lockwood. Cambridge: Cambridge University Press (1976)

Lockwood, A. P. M. and C. B. E. Inman: Diuresis in the amphipod *Gammarus duebeni* induced by methylmercury, D. D. T., lindane fenithrothion. Comp. Biochem. Physiol. *52C*, 75–80 (1975)

Maddreli, S. H. P. and J. E. Casida: Mechanism of insecticide - induced diuresis in *Rhodnius*. Nature, Lond. *231*, (5297), 55–56 (1971)

Millward, G. E. and J. D. Burton: Association of mercuric ions and humic acid in a sodium chloride solution. Mar. Sci. Comm. *1*(1), 15–26 (1975)

Moulder, S. M.: Effects of interactions between heavy metals and salinity on the physiological responses of the amphipod *Gammarus duebeni*, 174 pp. Ph.D. Thesis, University of Southampton. 1979

Murakami, T. H., M. Hayakawa, T. Fujii, T. Hara, Y. Itami, A. Kishida and I. Nisida: The effects of heavy metals on developing sea urchin eggs. J. Okayama Med. Soc. *88*, 39–50 (1976)

Pineda, J. P.: Aspects of the organic chemistry of sea water. M. Phil. Thesis, Southampton University, 1973

Pyefinch, K. A. and J. C. Mott: The sensitivity of barnacles and their larvae to copper and mercury. J. exp. Biol. *25*, 276–298 (1948)

Reeve, M. R., M. A. Walter, K. Darcy and T. Ikeda: Evaluation of potential indicators of sub-lethal toxic stress on marine zooplankton (feeding, fecundity, respiration and excretion): CEPEX. Bull. Mar. Sci. *27*(1), 105–113 (1977)

Tattersfield, F. and H. M. Morris: An apparatus for testing the toxic values of contact insecticides under controlled conditions. Bull. Entomol. Res. *14*, 223–233 (1924)

Williams, P. M.: The association of copper with dissolved organic matter in sea water. Limnol. Oceanogr. *14*, 156–158 (1969)

Date of final manuscript acceptance: August 19, 1980.
Communicated by J. Mauchline, Oban

EXHIBIT E

Marine Environmental Research **26** (1988) 161–179

The Toxicity of Paired Metal Mixtures to the Nematode *Monhystera disjuncta* (Bastian, 1865)

G. Vranken, C. Tiré & C. Heip*

Marine Biology Section, Zoology Institute, State University of Gent, Ledeganckstraat 35, B-9000 Gent, Belgium

(Received 28 March 1988; accepted 6 June 1988)

ABSTRACT

The toxicity of bipartite Hg, Cu, Zn and Ni metal mixtures was studied to the free-living marine nematode Monohystera disjuncta. *Observed mortality and developmental inhibition responses could not be predicted by independent dissimilar nor simple similar action. The toxic unit concept was also used to evaluate overall mixture toxicity. With regard to mortality all paired metal mixtures act in a less than additive manner. The developmental inhibition response was not so clear-cut: the Zn–Ni mixture acts synergistically; similarly the joint effect of Zn–Cu combinations and Cu–Ni mixtures, containing small amounts of Cu (1 mg litre^{-1}), act synergistically and the response type with regard to the Hg–Cu mixture is not very clear.*

INTRODUCTION

In the field, marine organisms are exposed to complex effluents, e.g. acid iron waste under varying environmental conditions. Therefore, it may seem rather surprising that most toxicological studies deal with dose–response relationships of single toxicants. With some groups of animals, however, such as free-living marine nematodes, our present knowledge even on the mode of action of single toxicants is very poor and at present virtually nothing is known about the toxicity of mixtures. Existing information on single species–single toxicant bioassays with a few nematode species, reveals that these organisms are relatively insensitive, particularly to heavy metals

* Present address: Delta Instituut Voor Hydrobiologisch Onderzoek, Vierstraat 28, 4 401 EA Yerseke (Zeeland), The Netherlands.

Marine Environ. Res. 0141-1136/89/$03·50

and some organics (Bogaert *et al.*, 1984; Vranken *et al.*, 1984; 1985; 1986; Vranken & Heip, 1986), although conflicting results exist (Howell, 1984). Even when the so-called sensitive biological criteria, such as fecundity and developmental inhibition, are used for toxicity ranking, minimum effective concentrations are situated in the lower ppm range.

Predicting environmentally safe limits from such single species–single toxicant assays, however, is highly questionable as the occurrence of interactive effects between the constituents of metal mixtures, both in a positive and negative sense has been reported (e.g. Sprague & Ramsay, 1965; Breittmayer & Guttierez-Galindo, 1981; Negilski *et al.*, 1981 and Howell, 1984). Recently, Hermens (1985) has shown that toxicity caused by complex mixtures, composed of organics with a similar mode of action, can be predicted on the basis of concentration-additivity (Hewlett & Plackett, 1979). This implies that hazard assessment based on the effects of single constituents may be deceptive (Hermens, 1985).

Several models have been proposed to tackle the study of the joint-action of mixtures. One of the most comprehensive approaches is that of Plackett & Hewlett (1952). They distinguished four different types of action among which were two non-interactive (independent dissimilar and simple similar action) and two interactive (complex similar and dependent dissimilar action) models. In the most simple case, the simple (parallel slopes for individual toxicants) similar action model, the response is completely predictable. For example, in an acute-toxicity assay, a mixture composed of two constituents will cause 50% mortality, when the concentrations of the substances are half as strong as their LC_{50}. In other words, the sum of the concentrations of the constituents when expressed as fractions of their LC_{50}, is always equal to 1. In the independent dissimilar action model, the expected response is again predictable. An organism receiving a mixture of two drugs, will only respond if one (or both) of the constituents is present at a concentration higher than the incipient lethal level. Thus in this case organisms will not respond at all at concentrations below the incipient lethal level. Hewlett & Plackett (1979) discussed three different models for independent action. When the tolerances of the organisms to the components of the mixture are positively correlated, the toxicity of the mixture is equal to the most toxic substance. When the tolerances of the organisms are uncorrelated, the proportion responding to a mixture of two drugs can be calculated according to the formula $P = 1 - (1 - P_1)(1 - P_2)$ (Finney, 1971), where P = proportion of organisms expected to respond to the mixture; P_1 and P_2 = percentage of individuals responding to each single toxicant. The expected response in the third model of independent action which assumes negative correlation of tolerances, is $P = P_1 + P_2$ or whichever is least.

However, predicting responses becomes more difficult when the constituents interact with one another. The toxicity of a mixture is then classified as partial-additive, synergistic and antagonistic (Könemann, 1981). Könemann (1981) proposed the use of a mixture toxicity index to evaluate the toxicity of equitoxic mixtures composed of more than two chemicals.

Another model which has been used frequently (Sprague & Ramsay, 1965; Negilski *et al.*, 1981; Broderius & Kahl, 1985) to study the effect of mixtures is the toxic-unit concept. This concept has been developed to predict the toxicity of mixtures by adding up the toxic effects of two or more toxicants acting at the same time. The concentration of each single constituent is now expressed in fractions of their incipient levels, which is the highest concentration giving a zero response and not in conventional chemical units (e.g. a concentration of a toxicant equally strong as the incipient level will be given a value of 1 toxic unit). After summing the toxic units assigned to each single constituent, the total toxic unit ascribed to the mixture can then be used to interpret its overall toxicity (Sprague & Ramsay, 1965). This concept consequently assumes that the components contribute in a similar way to the effect caused by the mixture. This implies that the toxic unit concept can be considered as a redefinition of the simple similar action model (Negilski *et al.*, 1981).

In this study we report on the toxicity of Cu/Hg, Zn/Ni, Zn/Cu and Cu/Ni mixtures to the nematode *Monhystera disjuncta*. Two criteria, mortality and developmental inhibition, were studied to evaluate the effect of the paired metal mixtures. We first examined whether the joint effect of the mixtures acted according to the toxic unit concept or according to two popular non-interactive models proposed by Plackett & Hewlett (1952). Between these two non-interactive models, a continuum of non-interactive models exists, which although not considered here are considered elsewhere (Negilski *et al.*, 1981; Broderius & Kahl, 1985). When none of these models matched the experimental figures interactive models were considered.

MATERIAL AND METHODS

Cultivation techniques and test procedures are completely identical to those described in our previous work (Vranken *et al.*, 1984, 1985). Briefly, the nematode, *Monhystera disjuncta*, is cultured in small petri-dishes filled with 0·5% sterile bacto-agar made up in artificial seawater according to Dietrich & Kalle (1957) with a salinity of 30‰. The agar was enriched with a sterol-mixture (Vanfleteren, 1980) and the bacterium *Alteromonas haloplanktis* is administered as food. The test procedure consists in sampling at random 120 J_2-larvae which were distributed equally among four replicates. After a

test-period of 96 h the number of dead juveniles and adults were counted. During the test period some worms left the agar bottoms and dried up. Such mortalities, and mortality caused by transferring the larvae from the stocks to the experimental cultures, were not included in the analysis. The experiments were run at 17°C in the dark. In the present assays no mortalities were observed in the controls.

STATISTICAL ANALYSIS

EC_{50} values for developmental inhibition were calculated by minimum logit Chi-square analysis (Hewlett & Plackett, 1979). Confidence limits around the EC_{50} values were corrected for heterogeneity when indicated by goodness of fit analysis (Table 1). The incipient lethal levels (ILL) and the no effect levels with regard to developmental inhibition (NEL) were obtained by inverse prediction from linearized dose–response relationships by transferring the percentages into angles (arc sin $\sqrt{p}$) (Sokal & Rohlf, 1981). ANCOVA and regression analysis through the origin with appropriate ANOVA (Steel & Torrie, 1960) were used to test the toxic unit (TU) concept. For toxicants with parallel dose–response curves expected mortalities according to the simple similar action model were estimated from the dose–response curves of the single metals by expressing the mixture concentrations into an equivalent concentration of one metal: $Z = Z_1 + pZ_2$ where Z_1 and Z_2 are concentrations of two metals and p is the relative potency ratio (Finney, 1971). Expected mortalities to test the independent dissimilar action were again calculated from the individual dose–response relationships. The tolerances of the nematodes to each single metal were considered to be uncorrelated. Both the independent dissimilar action model and the simple similar action model were tested by Chi-square goodness of fit analysis (Finney, 1971). The percentages of juveniles responding when developmental inhibition was used as the criterion were corrected for control response by Abbot's formula (Finney, 1971). The logits of 0% and 100% were calculated as logit ($\frac{1}{2}$ m) and logit ($1-\frac{1}{2}$ m) respectively (Hewlett & Plackett, 1979).

RESULTS

Developmental inhibition

EC_{50}s based on development inhibition for each single metal constituent of the mixtures tested are shown in Table 1. Using this criterion, Cu and Hg

TABLE 1

Monhystera disjuncta. Minimum Logit Chi-square Analysis of the Logit of the Percentage Juveniles (1) Against the Logarithm of the Concentration (*C*):

Metal	*t*	*f*(±*SE*)	EC_{50} (*95% CI*)	χ^2 (*df*)
Hg	−2·93	8·14 (±1·309)	2·3 (1·70–3·09)	12·6** (3)
Cu	−2·17	6·38 (±4·566)[a]	2·2 (0·025–193·160)	6·7** (1)
Zn	−9·34	12·40 (±1·668)	5·7 (5·26–6·09)	1·6 NS (1)
Ni	−7·20	5·51 (±0·507)	20·3 (18·25–22·49)	7·1 NS (3)

[a] Slope in NS ($t_s = 1{\cdot}573 < 12{\cdot}706$).

** ($0{\cdot}001 < P < 0{\cdot}01$).

$l = t + f \log_{10} C$; t = Intercept; f = slope; $m = EC_{50}$ in mg litre^{-1}; SE = standard error; CI = confidence interval; χ^2 = Chi-square for goodness of fit; NS = not significant.

appeared to be equitoxic. Zn is intermediate toxic and Ni is relatively non-toxic.

Copper mercury mixtures

Mortality. Observed mortalities of *Monhystera disjuncta* exposed to Cu/Hg mixtures were significantly different from expected mortalities based on both independent dissimilar action and simple similar action ($P < 0{\cdot}001$). The potency ratio (PR) used to express the Hg content of the mixture into an equivalent Cu concentration is estimated as PR = 0·431 (Table 2). The simple similar action model overestimated mortality in all cases (Table 3). At 1 combination (2·5 ppm Hg/1 ppm Cu) the observed mortality was higher when compared with the expected death rate according to the independent dissimilar action (Table 4). The incipient lethal levels (ILL) of Cu and Hg were determined as 0·7 and 1·1 mg litre^{-1}, respectively (Table 5). Percentage mortality (transformed into logits) caused by each single metal and by the mixture is plotted against the logarithms of the toxic units in Fig. 1a. The response curves of each single metal are almost identical (Fig. 1a); hence, a single line can be plotted through the data-points (Fig. 1a). The toxic units of the mixture varied between 2·3 and 5·8. They overestimated in all cases the overall mixture toxicity significantly (Fig. 1a, Table 6). The mixture with a strength of 3·7 TU containing, respectively, 2·5 mg litre^{-1} Hg and mg litre^{-1} Cu is more toxic than the mixture with a strength of 4·5 TU composed out of 1 mg litre^{-1} of the less toxic Hg and 2·5 mg litre^{-1} of the relatively more toxic Cu. As a result the curve depicting mortality against the TU of the mixture shows an indented pattern (Fig. 1a). From Fig. 1a it is obvious that the mixture's ILL is considerably higher than the ILL's of each single metal.

TABLE 2

Monhystera disjuncta. Combined Slopes (b) with Standard Error and Potency Ratios (PR) with 95% Confidence Limits for Cu/Hg, Zn/Ni, Zn/Cu and Cu/Ni 96 h Dose–Mortality Curves, χ^2 (Heterogeneity) Tests the Goodness of Fit, the Second χ^2 and/or the Variance Ratio Test, Examines Whether the Dose–Response Curves are Parallel; NP = Not Parallel

	Cu/Hg	*Zu/Ni*	*Zn/Cu*	*Cu/Ni*
b (±SE)	8·35 (0·842)[a]	7·17 (0·553)	NP	NP
PR (±95% CI)	0·431(0·388–0·497) (0·349–0·533)[a]	0·234 (0·209–0·263)		
χ^2 (heterogeneity)	15·75 (df = 6; P = 0·015)	7·44 (df = 5; P = 0·190)	11·83 (df = 6; P = 0·066)	8·17 (df = 5; NS)
χ^2 or F_s (test for parallelism)	6·24 (df = 1, 6; P = 0·047)	0·39 (df = 1; P = 0·531)	16·60 (df = 1; P < 0·001)	7·07 (df = 1; P = 0·008)

[a] Corrected for heterogeneity.

TABLE 3

Monhystera disjuncta. Concentrations of Bipartite Mixtures of Hg, Cu, Ni and Zn in mg litre^{-1}; Expected Mortalities Based on Simple Similar Action and Observed Mortalities

Mixture concentrations (mg litre^{-1})		*%Mortality expected by simple similar action*	*%Observed mortality*
Hg + Cu			
1	1	12·38	0
2·5	1	35·31	11·32
1	2·5	65·51	0
2·5	2·5	79·64	2·56
		χ^2: 694·2 ($P < 0{\cdot}001$)	
Ni + Zn			
5	1	0·05	0
5	5	1·32	0
2·5	1	1·83	0
5	10	7·84	0
2·5	10	20·22	5·10
		χ^2: 26·3 ($P < 0{\cdot}001$)	

Developmental inhibition. For this criterion only the independent dissimilar action model was tested as there exists significant heterogeneity ($P < 0{\cdot}001$) around the common Cu/Hg slope (Table 7). Expected numbers of juveniles lowering their development rate, as based on independent dissimilar action, were significantly different from the observed numbers ($P < 0{\cdot}001$; Table 8). Except for the 2·5 mg litre^{-1} Hg + 1 mg litre^{-1} Cu mixture the model overestimated developmental inhibition. The threshold NEL for developmental inhibition were estimated as 0·65 and 0·7 mg litre^{-1} for, respectively, Hg and Cu. The toxic units of the mixture are higher than 1. However, as the developmental inhibition (percentage individuals remaining juvenile) versus toxic unit plots of both single metals cannot be fitted by a single line (Table 9), the toxicity of the mixture is a function of the proportion of each constituent. Nevertheless, on statistical grounds no arguments exist to reject the toxic unit concept for mixtures possessing a strength of 1 TU (Table 10). As for mortality, the mixture composed proportionally out of more Hg, causes a greater effect on development.

Zinc–nickel mixtures

Mortality. The common slope and PR are given in Table 2. Observed and expected mortalities for both non-interactive models tested are significantly different (Tables 3 and 4). The independent dissimilar action model overestimated slightly the observed mortalities ($0{\cdot}05 > P > 0{\cdot}01$). Differences

TABLE 4

Monhystera disjuncta. Concentrations of Bipartite Mixtures of Hg, Cu, Ni and Zn in mg litre^{-1}; Expected Mortalities for Each Single Metal; Expected Mortalities According to Independent Dissimilar Action; Observed Mortalities and Toxic Units of the Mixture Concentrations

Mixture concentrations (mg litre^{-1})		*%Mortality expected for each single metal*		*%Mortality expected by independent dissimilar action*	*%Observed mortality*	*Toxic units*
Hg + Cu		Hg	Cu			
1	1	0·50	1·13	1·62	0	2·34
2·5	1	7·65	1·13	8·70	11·32	3·70
1	2·5	0·50	55·42	55·64	0	4·48
2·5	2·5	7·65	55·42	58·83	2·56	5·84
				$\chi^2 = 302{\cdot}5$ ($P < 0{\cdot}001$)		
Ni + Zn		Ni	Zn			
5	1	0·004	0·1	0·01	0	0·32
5	10	0·004	6·12	6·13	0	1·82
25	1	0·81	0·1	0·81	0	0·93
25	10	0·81	6·12	6·88	5·10	2·43
5	5	0·004	0·79	0·80	0	0·99
				$\chi^2 = 9{\cdot}0$ ($0{\cdot}05 > P > 0{\cdot}01$)		
Zn + Cu		Zn	Cu			
1	1	0·01	1·13	1·14	0	1·6
5	1	0·79	1·13	1·92	0	2·3
10	1	6·12	1·13	7·19	3·74	3·1
1	2·5	0·01	55·42	55·43	4·85	3·7
10	2·5	6·12	55·42	58·15	25·89	5·2
				$\chi^2 = 160{\cdot}0$ ($P < 0{\cdot}001$)		
Cu + Ni		Cu	Ni			
1	5	1·13	0·004	1·14	0	1·58
1	25	1·13	0·81	1·93	0	2·20
2·5	5	55·42	0·004	55·42	0	3·72
2·5	25	55·42	0·81	55·78	27·08	4·34
				$\chi^2 = 178{\cdot}4$ ($P < 0{\cdot}001$)		

TABLE 5

Monohystera disjuncta. Incipient Lethal Levels (ILL) in mg litre^{-1} with 95% CI and No Effect Levels (NEL) as Measured by Developmental Inhibition of Different Metals as Obtained from Mortality/Dose and Developmental Inhibition/Dose Response Curves

Metal	*Mortality ILL in mg litre^{-1}*	*Developmental inhibition NEL in mg litre^{-1}*
Hg	1·1 (95% CI: 0–3·26)	0·65 (95% CI: 0·022–2·042)
Cu	0·7 (95% CI: 0·106–1·745)	0·7 (95% CI: 0·103–1·638)
Zn	6·0 (95% CI: 3·4–9·2)	1·6 (95% CI: 0·294–3·569)
Ni	32·6 (95% CI: 10·9–47·9)	4·0 (95% CI: 0·625–10·64)

between observed and theoretical frequencies based on simple similar action are more pronounced which is expected because the response for similar action is always higher than the independent dissimilar action when the logit slopes $\geq 1{\cdot}43$ for the individual toxicants (Table 2) (Christensen *et al.*, 1985). The ILL of Zn and Ni are 6·0 and 32·6 mg litre^{-1}, respectively. The mortality TU curves for each single metal match and their plots can be fitted by a single curve (Fig. 1b). The mixture TU's ranged between 0·3 and 2·4. In two

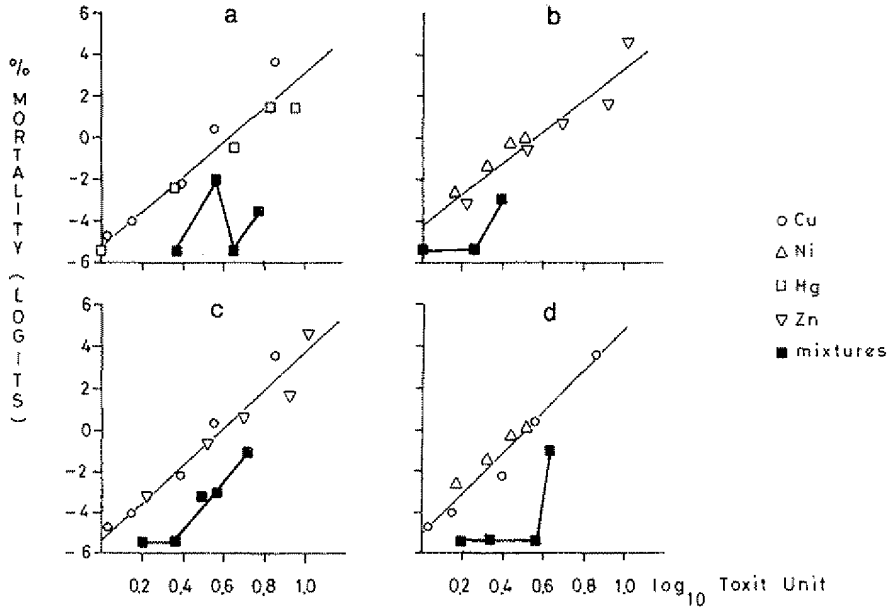

Fig. 1. *Monhystera disjuncta*: percentage mortality of juveniles exposed to Cu, Hg, Zn, Ni and paired mixtures of these metals.

TABLE 6
Monhystera disjuncta. Expected Mortalities Based on the Toxic Unit Concept Compared with Observed Mortalities

Mixture	*Toxic units*	*Expected mortality (%) and 95% CI*	*Observed mortality (%)*
Hg/Cu	2·3	10·7 (5·59–19·57)	0·0
	3·7	37·9 (23·24–55·08)	11·3
	4·5	54·6 (35·94–71·98)	0·0
	5·8	75·5 (55·76–88·22)	2·6
Zn/Ni	0·3	0·04 (0·01–0·29)	0·0
	0·9	1·15 (0·36–3·59)	0·0
	1·0	1·41 (0·47–4·19)	0·0
	1·8	9·7 (4·91–18·09)	0·0
	2·4	21·7 (13·32–33·28)	5·1
Zn/Cu	1·6	3·2 (1·5–6·55)	0·0
	2·3	12·1 (7·03–20·09)	0·0
	3·1	30·9 (20·82–43·29)	3·7
	3·7	47·4 (41·72–53·09)	4·9
	5·2	77·5 (65·52–86·22)	25·9
Cu/Ni	1·6	4·2 (2·38–7·22)	0·0
	2·2	15·7 (10·51–22·78)	0·0
	3·7	65·1 (51·21–76·86)	0·0
	4·3	78·6 (65·43–87·72)	27·1

combinations 5 mg litre^{-1} Ni + 5 mg litre^{-1} Zn and 25 mg litre^{-1} Ni 1 mg litre^{-1} Zn with a TU very close to 1, no mortality was observed. Nevertheless, the TU model overestimated the overall mixture toxicity (Table 6).

Developmental inhibition. The slopes of the curves depicting the numbers not maturing against the concentration of each single metal constituent of this mixture were not parallel. The independent dissimilar action model significantly underestimated the response ($P < 0{\cdot}001$) (Table 8). The threshold NELs for developmental inhibition were determined at 1·6 and 4·0 mg litre^{-1} for Zn and Ni, respectively. It seems that the toxicity of the mixture is more than additive (Fig. 2b). This apparent synergistic effect is not pronounced enough to be statistically significant (ANCOVA; $P = 0{\cdot}087$).

Zinc–copper mixtures

Mortality. Observed mortalities over the entire range tested were significantly less ($P < 0{\cdot}001$) than the expected death rate based on

TABLE 7

Monhystera disjuncta. Combined Slopes (b) with Standard Error and Potency Ratios (PR) with 95% Confidence Limits for Cu/Hg, Zn/Ni, Cu/Zn and Cu/Ni Dose–Developmental Inhibition Curves; χ^2 and F_s as in Table; NP = Not Parallel

	Cu/Hg	*Zn/Ni*	*Cu/Zn*	*Cu/Ni*
b (±SE)	7·88 (1·30)	NP	9·20 (2·33)	5·59 (0·90)
PR (±95% CI)	0·872 (0·549–1·771)		0·330 (0·166–0·657)	0·115 (0·071–0·240)
χ^2 (heterogeneity)	19·31 (df = 4, $P < 0·001$)	8·73 (df = 4; NS)	8·34 (df = 2; $P = 0·015$)	13·79 (df = 4; $P = 0·008$)
χ^2 or F_s (test for parallelism)	0·223 ($f = 1, 4$; NS)	15·63 (df = 1, $P < 0·001$)	1·66 (df = 1,2; NS)	< 1; NS

TABLE 8

Monhystera disjuncta. Concentrations of Bipartite Mixtures of Hg, Cu, Ni, and Zn in mg litre^{-1}; Observed % Juveniles for each Single Metal; Expected % Juveniles According to Independent Dissimilar Action; Observed % Juveniles, Corrected for Control Response with the Formulae $(P-C)/(1-C)$ (Hewlett & Plackett, 1979), and Toxic Units

Mixture concentrations (mg litre^{-1})		*% Juveniles responding for each single metal*		*% Juveniles expected by independent dissimilar action*	*% Juveniles observed*	*Toxic units*
Hg + Cu		Hg	Cu			
1	1	3·18	10·96	13·79	7·47	3·0
2·5	1	71·30	10·96	74·45	99·49	5·3
1	2·5	3·18	99·31	99·33	87·04	5·1
2·5	2·5	71·30	99·31	99·80	99·08	7·4
				χ^2: 77·8 ($P < 0·001$)		
Ni + Zn		Ni	Zn			
5	1	4·42	0	4·42	4·62	1·9
5	10	4·42	97·24	97·36	99·36	7·5
25	1	57·35	0	57·35	99·40	6·9
25	10	57·35	97·24	98·82	99·33	12·5
5	5	4·42	33·28	36·23	96·81	4·4
				χ^2: 190·5 ($P < 0·001$)		
Zn + Cu		Zn	Cu			
1	1	0	10·96	10·96	30·43	2·1
5	1	33·28	10·96	40·59	99·53	4·6
10	1	97·24	10·96	97·54	99·48	7·7
1	2·5	0	99·31	99·31	99·46	4·2
10	2·5	97·24	99·31	99·98	99·50	9·8
				χ^2: 67·0 ($P < 0·001$)		
Cu + Ni		Cu	Ni			
1	5	10·96	4·42	14·90	53·54	2·7
1	25	10·96	57·35	62·02	99·41	7·7
2·5	5	99·31	4·42	99·34	99·47	4·8
2·5	25	99·31	57·35	99·71	99·37	9·8
				χ^2: 23·8 ($P < 0·001$)		

Slopes of Cu and Ni are not significantly different from zero, therefore experimental percentages have been used to determine expected frequencies.

TABLE 9

Monhystera disjuncta. Linear Least Squares Unweighted Regression Analysis of the Logit of the Percentage Juveniles Responding (1) Against the Logarithm of the Toxic Units (TU) of single Metals and Mixtures $1 = a + b \log_{10} TU$; a = Intercept; b = Slope: r^2 = Coefficient of Determination; F_s = Variance Ratio Testing the Significance of Regression; P = Probability

Metals	*a(SE)*	*b (95% CI)*	r^2	F_s	*P*
Hg	−4·76 (0·68)	8·47 (2·50)	0·97	116·3	0·002
Cu	−5·44 (3·45)	16·66 (108·70)	0·79	3·8	0·302
Zn	−5·35 (1·93)	10·20 (29·5)	0·95	19·3	0·143
Ni	−4·53 (1·40)	7·41 (5·69)	0·85	17·2	0·025
Cu/Hg	−11·03 (4·87)	19·27 (30·56)	0·80	7·85	0·107
Zn/Ni	−6·40 (1·26)	13·76 (8·03)	0·97	57·9	0·017
Zn/Cu	−6·57 (0·77)	18·48 (17·59)	0·99	178·3	0·048
Cu/Ni	−8·41	19·98			

independently dissimilar action. The dose–mortality curves of Zn and Cu are not parallel. The TU concept significantly overestimated toxicity (Table 6; Fig. 1c) both at a mixture strength equal to 1 TU ($0·01 < P < 0·05$) and greater than 1 TU (ANCOVA; $P < 0·001$). Thus Zn and Cu when together, act in a less than additive manner. The lower toxicity of the mixture when compared with the single metals is clearly shown in Fig. 1c.

TABLE 10

Monhystera disjuncta. F-test Examining Whether the Regression of the Numbers Responding, Corrected for Control Response and Transformed to Arc sin $\sqrt{p}$, Against the Logarithm of the Toxic Units of Metal Mixtures, Passes through the Origin. When the Intercept of this Relationship is Significantly Different from Zero, as Indicated by a High F_s-value, Metal Mixture Concentrations Corresponding with a Toxic Unit = 1 Induce Mortalities Significantly Different From the Expected Zero Response

	Metal mixture			
Criterion	Cu/Hg	Zn/Ni	Zn/Cu	Cu/Ni
Mortality	CR	ND	$F_s = 22·3$ (df = 1, 2; $0·01 < P < 0·05$)	ND
Development	$F_s = 2·76$ (df = 1, 2; NS)	$F_s = 0·63$ (df = 1, 2; NS)	$F_s = 4·09$ (df = 1, 1; NS)	ND

ND: not determinable from the present data set.
CR: curvilinear relationship.
df = degrees of freedom.

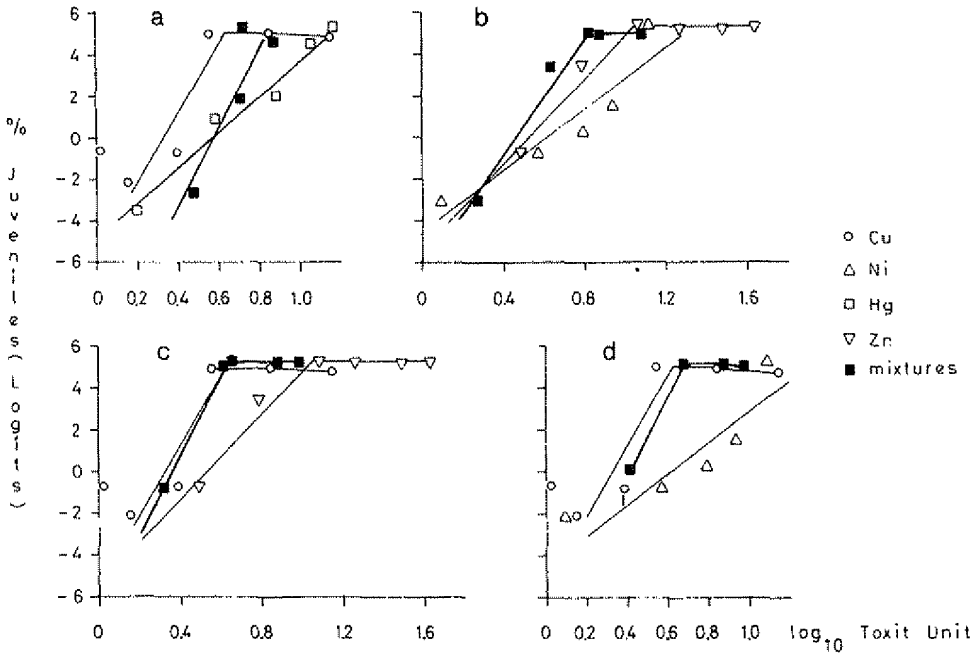

Fig. 2. *Monhystera disjuncta*: percentage worms remaining juvenile after exposure to Cu, Hg, Zn, Ni and paired mixtures of these metals.

Developmental inhibition. The number of worms not maturing were not predictable by independent dissimilar action ($P < 0{\cdot}001$). At two mixture concentrations—1 mg litre^{-1} Zn + 1 mg litre^{-1} Cu and 5 mg litre^{-1} Zn + 1 mg litre^{-1} Cu—the observed numbers remaining juveniles were twice as high as expected. Simple similar action might result in biased predictions as there exists significant heterogeneity around the combined Zn/Cu curves (Table 2). Especially at higher Cu concentrations (Cu < 1·75 mg litre^{-1}) predictions based on the logit-response curve are unreliable. For mixtures containing 1 mg litre^{-1} Cu more or less reliable expected frequencies can be determined by using the simple similar action model. The expected percentage worms remaining juvenile are for the three combinations tested, containing 1 mg litre^{-1} Cu (namely, 1 mg litre^{-1} Zn + 1 mg litre^{-1} Cu, 5 mg litre^{-1} Zn + 1 mg litre^{-1} Cu and 10 mg litre^{-1} Zn + 1 mg litre^{-1} Cu) less than those observed (20% expected versus 30% observed; 63% expected versus 99·5% observed and 87% expected versus 99·5% observed). The response curve of the mixture lies very close to that of Cu, when the metal amount is expressed in TU's. This, together with the fact that simple similar action underestimates developmental inhibition, reveals that the overall mixture toxicity is more than expected from independent dissimilar joint action (Table 8).

Copper–nickel mixtures

Mortality. The independent dissimilar action model overestimated in all mixture combinations tested the observed mortalities ($P < 0{\cdot}001$). In two mixtures containing copper concentrations higher than the 96 h LC_{50}, observed mortality was significantly less ($P < 0{\cdot}01$) when compared with expected mortalities caused by copper alone. The dose–mortality curves of Cu and Ni are not parallel (Table 2). The TU mortality curves of both single metals were fitted conveniently by one single curve (Fig. 1c). The TU overestimated in all combinations the overall toxicity of the mixture (Table 6). Again, the TU mortality curve lies completely under the curve depicting the response of each single metal (Fig. 1c). Therefore, Cu and Ni when together act in a less than additive manner.

Developmental inhibition. Observed percentages not reaching adulthood were significantly different from those predicted by independent dissimilar action ($P < 0{\cdot}001$). At 1 mg litre^{-1} Cu + 5 mg litre^{-1} Ni and 1 mg litre^{-1} Cu + 25 mg litre^{-1} Ni the observed % juveniles was considerably higher than expected. At these combinations the response type of the mixture appears to be more than additive as expected frequencies according to simple similar action were smaller than those observed (29% expected versus 54% observed and 77% expected versus 99% observed). Toxic units ranged between 2·7 and 9·8. As the TU developmental inhibition curve of both single metals cannot be fitted by one single line, the interpretation of the mixture toxicity based on the toxic unit concept is rather difficult.

DISCUSSION

Knowledge of the mode of toxic action of heavy metals to nematodes is very poor. Therefore we do not know which of the non-interactive models proposed by Plackett & Hewlett (1952), simple similar action or independent dissimilar action, is the more relevant. According to several authors (Barnes & Stanburry, 1948; Corner & Sparrow, 1956 and Negilski *et al.*, 1981) toxicants are acting on a similar site when the dose mortality curves of the single metals are parallel. Recently Christensen *et al.* (1985) have shown that parallel dose–response curves are not required for concentration addition (similar systems affected). Nevertheless, according to the present results one might expect, when mortality is considered as a criterion, that Cu and Hg on the one hand and Zn and Ni on the other possess a similar mode of action. For the developmental assay no clear-cut conclusions could be drawn concerning parallelism of the response curves as there exists high variability around the response curve of Cu. However, the design of acute and sublethal

toxicity tests only allows one to determine the probability distribution of lethal/sublethal threshold values and to estimate its central tendency (LC_{50} or EC_{50} value) and the measure of dispersion of the variables around the central tendency (standard deviation). Therefore, it is not obvious that the slope of the dose–response curve of a species to a particular chemical yields information concerning the mode of action of the chemical tested. The assumption of parallelism of slopes of the single mixture constituents is only required to obtain unbiased estimates of the expected frequencies according to the simple similar action model. Neither observed mortality responses nor developmental inhibition of juvenile *Monhystera disjuncta* could be predicted on the basis of independent dissimilar or simple similar action. Both models significantly overestimated the mortality response, except for the 2·5 mg litre^{-1} Hg + 1 mg litre^{-1} Cu mixture when the independent dissimilar action model underestimated the observed response of the bipartite metal mixture. The toxic unit mortality response curves of all single metals can be presented by one single line. Consequently, when mortality is considered, the toxic unit concept can also be used to evaluate the overall mixture response. The toxic unit concept overestimated mortality in all paired combinations. As the overall effect of the bipartite mixtures could not be determined from the dose–mortality curves of the single metals, we have concluded that all paired metal mixtures studied act in a less than additive manner. When developmental inhibition is considered, neither of the single metal toxic unit response curves overlap. This implies that the slopes of the individual dose–response curves are different. From the present developmental assays with *Monhystera disjuncta* we conclude that Zn–Ni mixtures act synergistically, that the joint action of three Zn–Cu combinations and two Cu–Ni mixtures, containing 1 mg litre^{-1} Cu, shows a similar effect and that the response type of the Hg–Cu mixture is not very clear. Similarly, as with mortality the mixture containing less copper is the most toxic. The reason why a different response type is observed for the two criteria tested is not known.

At low mixture concentrations the overall toxicity of the mixture is less than additive, which implies that the ILL in terms of toxic units of the mixture is higher when compared with the ILL of the single metals. This result is in agreement with findings reported in previous studies (Lloyd & Orr, 1969; Negilski *et al.*, 1981). Negilski *et al.* (1981) even found that mortalities were unimportant at concentrations of mixture constituents as high as one-third of the 14-day LC_{50} values.

Concerning mortality similar results were obtained by Negilski *et al.* (1981) who showed that the toxicity of Zn–Cu mixtures to the shrimp *Callianassa australiensis* was less toxic than was expected for the independent model. Mixtures of cadmium and copper (not studied in the

present assays) revealed a reverse effect and were more toxic than the independent model, but less toxic than expected by simple similar action. Sprague & Ramsay (1965) found that copper–zinc mixtures acted synergistically juvenile Atlantic salmon *Salmo salar*, which is in contrast with our results. Copper–mercury mixtures showed clear synergism, towards the harpacticoid *Nitocra spinipes* (Barnes & Stanburry, 1948), the copepod *Acartia clausi* and the brine shrimp *Artemia salina* (Corner & Sparrow, 1956), which again is in variance with the present results. These and other workers (Peyfinch & Mott, 1948; Hunter, 1949) believed that Hg and Cu acted differently. They thought that the toxic action of Cu basically acts indirectly by interfering with the respiratory enzyme system and/or the osmoregulatory system while they thought that mercury was acting directly by poisoning the protoplasma. Now it is known that mercury might interfere with any enzyme containing an SH group essential for its catalytic activity (Boudou *et al.*, 1983). Further Hunter (1949) reported the same observation as we did, namely, that small amounts of Cu enhanced the toxicity of mercury towards the amphipod *Marinogammarus marinus*, whereas small amounts of mercury did not affect copper toxicity.

More recently, Bræk *et al.* (1976) found that Cu–Zn mixtures acted synergistically towards three common phytoplankton species, whereas the same mixture showed antagonism when added to cultures of *Phaeodactylum tricornutum*. It was proposed that the antagonistic effect was caused by competition between the metals for a common uptake site. This hypothesis was substantiated by the fact that Zn^{2+} toxicity increased significantly when the magnesium content of the medium was reduced, which suggests that divalent metal cations act at a common site in *P. tricornutum*. Further, Christensen *et al.* (1985) reported that the joint action of Ni and Zn on *Selenastrum caprocornutum* is of the concentration additive type. Therefore, without giving a complete literature review concerning the joint action of metals, it is clear that all possible types of action were reported in bioassays in laboratory conditions.

The main problem, however, with this type of experiment is that the biochemical pathway and the mode of action of the chemicals with regard to the test organism is very poorly known. For instance, with nematodes, only one study discusses cadmium metabolism in relation to tissue distribution and accumulation by metal-binding proteins (Howell & Smith, 1985).

At present we only can state, as is generally accepted (Babich & Stotzky, 1983), that antagonistic interactions result from competition between the chemicals for sites on the cell surfaces, whereas synergism is indicative for an increased permeability of the plasma membrane. How this knowledge can be used to explain the difference in response type between the two criteria studied, is not known.

ACKNOWLEDGEMENTS

This research was supported through grant ENV-767-B of the Environmental Programme of the CEC. C.H. acknowledges a grant from the Belgian National Fund for Scientific Research.

REFERENCES

Babich, H. & Stotzky, G. (1983). Influence of chemical speciation on the toxicity of heavy metals to the microbiota. In *Aquatic Toxicology*, ed. J. O. Nriagu, John Wiley & Sons, New York, pp. 1–46.

Barnes, H. & Stanburry, F. A. (1948). The toxic action of copper and mercury salts both separately and when mixed on the harpacticoid copepod, *Nitocra spinipes* (Boeck). *J. Exp. Biol.*, **25**, 270–5.

Boudou, A., Georgescauld, D. & Desmazes, J. P. (1983). Ecotoxicological role of the membrane barriers in transport and bioaccumulation of mercury compounds. In *Aquatic Toxicology*, ed. J. O. Nriagu, John Wiley & Sons, New York, pp. 117–37.

Bogaert, T., Samailoff, M. S. & Persoone, G. (1984). Determination of the toxicity of four heavy metal compounds and three carcinogens using two marine nematode species, *Monhystera microphthalma* and *Diplolaimelloides bruciei*. In *Ecotoxicological Testing for the Marine Environment*, ed. G. Persoone, E. Jaspers & C. Claus, State Univ. Ghent and Inst. Mar. Scient. Res. Bredene, belgium, pp. 21–30.

Bræk, G. S., Jensen, A. & Mohus, Å (1976). Heavy metal tolerance of marine phytoplankton III. Combined effects of copper and zinc ions on cultures of four common species. *J. exp. mar. Biol. Ecol.*, **25**, 37–50.

Breittmayer, J.Ph. & Gutierrez-Galindo, E. A. (1981). Toxicité aigue d'associations de mercure et de zinc vis-à-vis la moule *Mytilus edulis* (L.). *Chemosphere*, **10**, 795–8.

Broderius, S. & Kahl, M. (1985). Acute toxicity of organic chemical mixtures to the fathead minnow. *Aquat. Toxicol.*, **6**, 307–22.

Christensen, E. R., Chen, C.-Y. & Kannall, J. (1985). The response of aquatic organisms to mixtures of toxicants. *Wat. Sci. Techn.*, **17**, 1445–6.

Corner, D. S. & Sparrow, B. W. (1956). The modes of action of toxic agents. I. Observations on the poisoning of certain crustaceans by copper and mercury. *J. Mar. Biol. Ass. U.K.*, **35**, 531–48.

Dietrich, G. & Kalle, K. (1957). Allgemeine Meereskunde. Eine Einfürung in die Ozeanographie Gebrüder Borntraeger, Berlin, Nikolassee.

Finney, D. J. (1971). *Probit Analysis*. Cambridge University Press, 333 pp.

Hermens, J. (1985). Effecten van complexe mengsels van waterverontreinigende stoffen. *Vakbl. Biol.*, **65**, 29–34.

Hewlett, P. S. & Plackett, R. L. (1979). *The Interpretation of Quantal Responses in Biology*. Edward Arnold Publishers, London, 82 pp.

Howell, R. (1984). Acute toxicity of heavy metals to two species of marine nematodes. *Mar. Environ. Res.*, **11**, 153–61.

Howell, R. (1985). Effect of zinc on cadmium toxicity to the amphipod *Gammarus pulex*. *Hydrobiologia*, **123**, 245–9.

Howell, R. & Smith, L. (1985). Flux of cadmium through the marine nematode *Enoplus brevis* Bastian, 1865. *Revue Nématol.*, **8**, 45–51.

Hunter, R. W. (1949). The poisoning of *Marinogammarus marinus* by cupric sulphate and mercuric chloride. *J. Exp. Biol.*, **26**, 113–24.

Könemann, H. (1981). Fish toxicity tests with mixtures of more than two chemicals: A proposal for a quantitative approach and experimental results. *Toxicology*, **19**, 229–38.

Lloyd, R. & Orr, L. D. (1969). The diuretic response by rainbow trout to sublethal concentration of ammonia. *Wat. Res.*, **3**, 335–44.

Negilski, D. S., Ahsanullah, M. & Mobley, M. C. (1981). Toxicity of zinc, cadmium and copper to the shrimp *Callianassa australiensis*. II. Effects of paired and triad combinations of metals. *Mar. Biol.*, **64**, 305–9.

Plackett, R. L. & Hewlett, P. S. (1952). Quantal responses to mixtures of poisons. *J. R. Statist. Soc. B*, **14**, 141–63.

Peyfinch, K. A. & Mott, J. C. (1948). The sensitivity of barnacles and their larvae to copper and mercury. *J. Exp. Biol.*, **25**, 276–98.

Sokal, R. R. & Rohlf, F. J. (1981). *Biometry*. Freeman and Company, San Francisco, 859 pp.

Sprague, J. B. & Ramsay, B. A. (1965). Lethal levels of mixed copper–zinc solutions for juvenile salmon. *J. Fish. Res. Bd. Canada*, **22**, 425–32.

Steel, R. G. D. & Torrie, J. H. (1960). *Principles and Procedures of Statistics*. McGraw-Hill Book Company, New York, 481 pp.

Vanfleteren, J. R. (1980). Nematodes as nutritional models. In *Nematodes as Biological Models*, Vol. 2, ed. B. M. Zuckerman, Academic Press, New York, pp. 47–79.

Vranken, G. & Heip, C. (1986) Toxicity of copper, mercury and lead to a marine nematode. *Mar. Poll. Bull.*, **17**, 453–7.

Vranken, G., Vanderhaeghen, R. & Heip, C. (1985). Toxicity of cadmium to free-living marine and brackish water nematodes (*Monhystera microphthalma*, *Monhystera disjuncta*, *Pellioditis marina*). *Dis. Aquat., Org.* **1**, 49–58.

Vranken, G., Vanderhaeghen, R. Van Brussel, D., Heip, C. & Hermans, D. (1984). The toxicity of mercury to the free-living marine nematode *Monhystera disjuncta* Bastian, 1865. In *Ecotoxicological Testing for the Marine Environment*, ed. G. Persoone, E. Jaspers & C. Claus, State Univ. Ghent and Inst. Mar. Scient. Res. Bredene, Belgium, pp. 271–91.

Vranken, G., Vanderhaeghen, R., Verschraegen, K. & Heip, C. (1986). Toxicity of environmental toxicants on the marine nematode *Monhystera disjuncta*. A comparison between developmental rate, fecundity and mortality as toxicity-indices. Report to the CEC, 18 pp.

EXHIBIT F

J. exp. mar. Biol. Ecol., 1976, Vol. 25, pp. 37–50; © North-Holland Publishing Company

HEAVY METAL TOLERANCE OF MARINE PHYTOPLANKTON. III. COMBINED EFFECTS OF COPPER AND ZINC IONS ON CULTURES OF FOUR COMMON SPECIES

GUDMUND SKJÅK BRÆK AND ARNE JENSEN

Institute of Marine Biochemistry, University of Trondheim, Trondheim, Norway

and

ÅGE MOHUS

Nordland Regional College, Bodø, Norway

Abstract: The combined effects of copper and zinc ions on the growth of three marine diatoms and one dinoflagellate in culture have been studied. The two metals were found to act synergistically to all algae except *Phaeodactylum tricornutum* Bohlin. With this species an antagonistic effect was observed. Addition of zinc ions reduced the inhibition of growth caused by the more toxic copper ions. Zinc toxicity to this alga increased at low concentration of magnesium, indicating a common route for divalent metal ions in general.

INTRODUCTION

While our knowledge of the toxicity of single species of heavy metals to algae is increasing quite rapidly, very few studies have been concerned with combined effects of two or more metals, despite the well known fact that heavy metals almost always interact in natural waters. Hutchinson (1974) found that copper and nickel ions acted synergistically and that selenium and cadmium ions showed antagonism when added to cultures of freshwater green algae. Bartlett, Rabe & Funk (1974) studied effects of combinations of copper, zinc and cadmium on the freshwater chlorophyte *Selenastrum capricornutum*. No investigations on the combined effect of heavy metals on marine microalgae have been found in the literature.

The present work is part of a systematic study of the toxic effect of heavy metals on marine algae; the two first parts (Jensen, Rystad & Melsom, 1974, 1976) were concerned, respectively, with the effects of zinc and copper ions on growth and metal uptake of marine phytoplankton cultivated in fjord water. Studies on the toxic effect of these metals applied jointly have now been carried out on cultures of marine algae, and the results are collected in the present paper.

MATERIALS AND METHODS

The algae used were: *Phaeodactylum tricornutum* Bohlin (unialgal) obtained by the courtesy of Dr R. R. L. Guillard and Dr J. H. Ryther, Woods Hole, Mass., U.S.A.; *Skeletonema costatum* (Grev.) Cleve, clone Skel-5 (unialgal, axenic) isolated

by S. Myklestad, Institute of Marine Biochemistry, Trondheim, Norway; *Skeletonema costatum* (Grev.) Cleve, clone Skel-0 (unialgal, axenic) isolated by S. Myklestad; *Thalassiosira pseudonana* (Huds.) Hasle (unialgal, axenic) obtained by the courtesy of Dr Guillard and Dr Ryther; and *Amphidinium carteri* Hulburt (unialgal) isolated by J. Throndsen, Institute of Marine Biology, University of Oslo, Norway. The media used were:

i) The f medium of Guillard & Ryther (1962) in the 1/10 dilution with sea water and 25 % reduced salinity (designation 3/4 f/10).

ii) The same medium as above without the chelator (EDTA) and the trace mineral mixture (designated 3/4f/10-EDTA).

iii) Synthetic sea-water medium (designation L/10). This medium was used in the experiments with varying magnesium concentrations and was a modification of that of Lewin & Lewin (1967). The content of nitrate, phosphate, silicate, vitamins and micro-minerals corresponded to those of the f/2 medium, except for Fe and EDTA which were reduced to 10 % of the f/2 strength. Sulphate was added as Na_2SO_4 instead of $MgSO_4$ and the magnesium concentration varied by addition of $MgCl_2 . 6H_2O$ between 2.5 and 80 mM.

The media were sterilized either by autoclaving or by filter sterilization (0.2 μm membrane filter).

Heavy metal solutions were made by dissolving copper and zinc sulphate separately in distilled water to give a final concentration of 1 mg of metal/ml of solution. To each 1 l of these stock solutions 10 drops of concentrated HCl were added to prevent precipitation. Magnesium chloride was used for the magnesium solution. Suitable volumes of the stock solutions were added to test cultures immediately prior to inoculation.

Stock cultures of the various algae were maintained in f medium diluted to half strength (f/2).

All experiments were carried out in Erlenmeyer flasks which had been coated with silicone (Erickson, Lackie & Maloney, 1970). Exponentially growing cells were used for inoculation to give initial cell densities between 2.10^4 and 4.10^4 cells/ml of culture. The experiments were carried out at 13 °C (± 1 %) using cool white fluorescent light, ≈ 2600 lux in a light/dark regime of 14 h/10 h. Growth of the cultures was followed by counting in a Celloscope model 41 (for *P. tricornutum*, *T. pseudonana* and *A. carteri*) or under the microscope using a haemocytometer (Skel-0 and Skel-5). The cultures were followed for 10–14 days, and the growth rates calculated for the most active part of the logarithmic growth phase.

Results

Combined effect of copper and zinc on the growth of *Amphidinium carteri*

The data in Table I show that the growth rate of this species was unaffected by zinc ions in concentrations up to 200 μg/l, but there was a significant reduction in

TABLE I

Growth rate of *Amphidinium carteri* (divisions/day) at various concentrations of Zn and Cu in the medium: d, dead; * dead after 1 division.

Zn added, μg/l →	Cu added, μg/l → 0	50	75	100	150	200	250
0	1.7	1.1	0.8	0.7	0.6	0.3	d
50	1.7	0.8	0.6	0.3			
100	1.6	0.8	0.4	d*			
200	1.4	0.6	d				
400	1.1						
500	1.0						
1000	0.4						
2000	0.2						

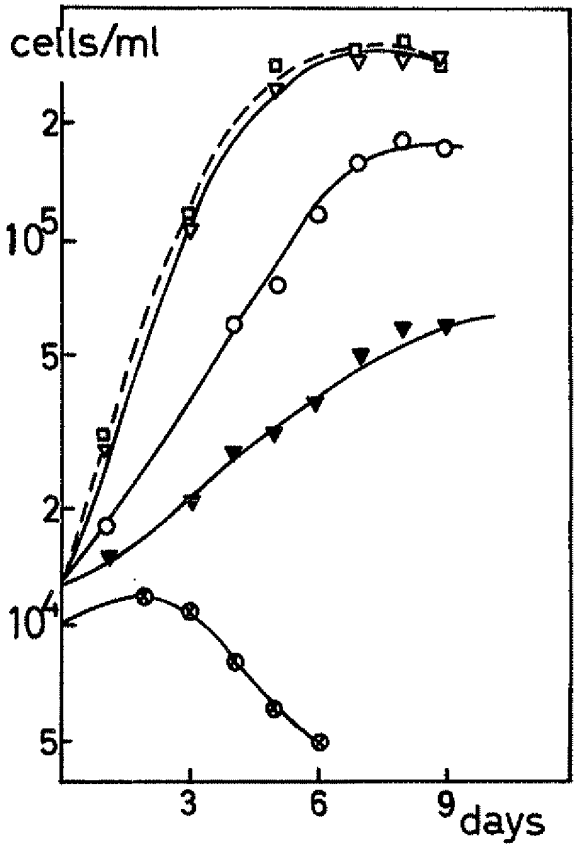

Fig. 1. Growth curves of cultures of *Amphidinium carteri* at various concentrations of Zn and Cu: □, control; ▽, 100 μg Zn/l; ○, 75 μg Cu/l; ▼, 100 μg Zn+75 μg Cu/l; ⊗, 200 μg Zn+75 μg Cu/l.

TABLE II

Growth rate of *Thalassiosira pseudonana* (divisions/day) at various concentrations of Zn and Cu in the medium.

		Cu added, µg/l →			
		0	50	100	200
Zn added, µg/l ↓	0	1.8	1.5	1.2	d*
	100	1.8		0.7	
	200	1.8		0.5	
	400	1.4		d*	
	500	0.7			

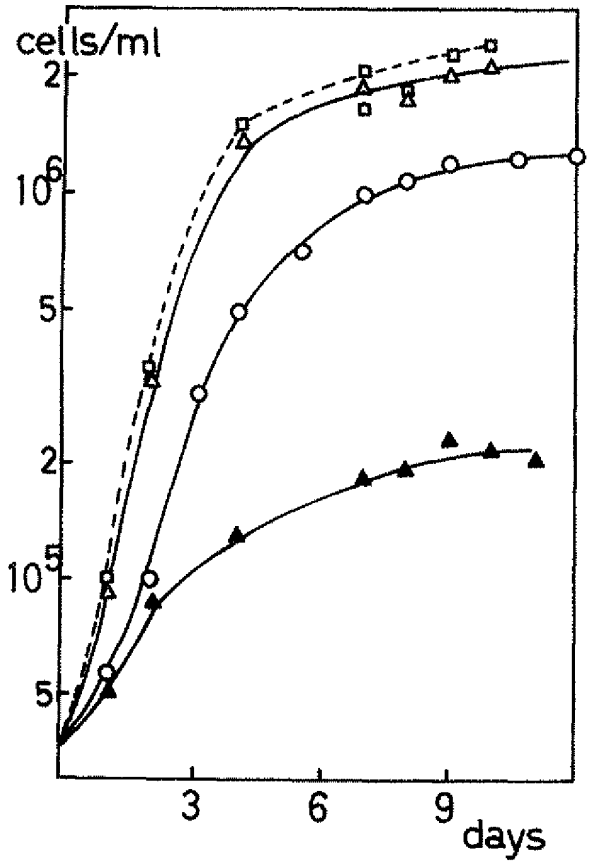

Fig. 2. Growth curves of cultures of *Thalassiosira pseudonana* at various concentrations of Zn and Cu: □, control; △, 200 µg Zn/l; ○, 100 µg Cu/l; ▲, 200 µg Zn+100 µg Cu/l.

the growth rate with 400 μg zinc/l. The cells grew, however, well at this concentration, and even 2000 μg zinc/l did not kill the cells. Addition of copper alone was considerably more toxic than zinc applied singly since there was a significant reduction in growth rate at a copper concentration of 75 μg/l. The lethal concentration was found to be ≈ 250 μg/l.

Table I further shows that the combination of copper and zinc ions was clearly more toxic than the metals applied singly. Addition of 75 μg copper/l alone gave 0.8 divisions/day and 100 μg zinc/l gave no reduction in growth rate. When these two treatments were combined, the growth rate was reduced to 0.4 divisions/day. This typical case of synergism is very clearly seen in Fig. 1, which also shows that there were no problems with extended lag periods during these experiments.

COMBINED EFFECTS OF COPPER AND ZINC ON THE GROWTH OF *THALASSIOSIRA PSEUDONANA*

The results are given in Table II (growth rates in the exponential phase) and in Fig. 2 (growth curves of control and three test cultures). This alga tolerated 200 μg Zn/l in the culture medium with no signs of reduced division rate, while the same concentration of copper was lethal.

Combinations of zinc and copper gave a synergistic response as with *A. carteri*. This is clearly seen in Fig. 2, which again indicates no extension of the lag period upon application of heavy metals.

COMBINED EFFECTS OF COPPER AND ZINC ON THE GROWTH OF *SKELETONEMA COSTATUM*

Data from the growth experiments carried out with the two clones of *S. costatum* are collected in Tables III and IV. Skel-0 is a zinc tolerant clone and this organism survived exposure to 400 μg zinc/l moderately well, while the clone from the Trondheimsfjord (Skel-5) stopped growing beyond the first division in the medium which contained 200 μg zinc/l. Copper was very toxic to both clones, the Skel-0 clone being somewhat more resistant than the Skel-5 clone. Applied in combination the two

TABLE III

Growth rate of *Skeletonema costatum* clone Skel-0 (divisions per day) at various concentrations of Zn and Cu in the medium: d, no division.

		Cu added, μg/l →				
		0	50	75	100	150
Zn added, μg/l →	0	1.3	1.2	1.0	1.0	0.5
	50	1.3			1.0	
	100	1.3			0.7	
	200	0.6			0.5	
	400	0.5			d	

metals gave synergistic effects in most cases. Due to the fact that *Skeletonema* was the least tolerant species, the results of these experiments were not so clear-cut as those obtained for the other algae tested.

TABLE IV

Growth rate of *Skeletonema costatum* clone Skel-5 (divisions/day) at various concentrations of Zn and Cu in the medium: * one division only; ** dead after 2 divisions.

Zn added, μg/l →	Cu added, μg/l → 0	50	75	100	150
0	1.1	1.0	0.7	0.6	0.3*
50	1.1			0.5	
100	0.9			**	
200	0.3*			d	
400	0.2*				

COMBINED EFFECTS OF COPPER AND ZINC ON THE GROWTH OF *PHAEODACTYLUM TRICORNUTUM*

Cultures of *P. tricornutum* were grown in media with and without the chelator (EDTA), and copper and zinc ions were added systematically. The results obtained are given in Tables V and VI: in the latter Table the divisions/day of single cultures are given together with the mean values for the series to demonstrate the accuracy attained under rigidly standardized conditions.

TABLE V

Growth rate of *Phaeodactylum tricornutum* (divisions/day) at various concentrations of Zn and Cu in the medium 3/4 f/10-EDTA (average of 2–4 parallell cultures).

Zn added, μg/l →	Cu added, μg/l → 0	250	500	750	1000
0	0.8	0.3	0.2	0.1	0.1
1000	0.9	0.5			
2000	0.9	0.5			
4000	0.8	0.4			
5000	0.7	0.4			
8000	0.6	0.4			
10,000	0.6	0.3			
50,000	0.1				

Fig. 3 shows the effect of zinc addition on growth curves of *P. tricornutum* in the 3/4 f/10 medium, demonstrating increasing reduction in growth rate with increasing zinc concentration in the medium. No effect on the final cell yield was obtained even on addition of 4000 μg zinc/l of medium. The effect of copper added alone and in

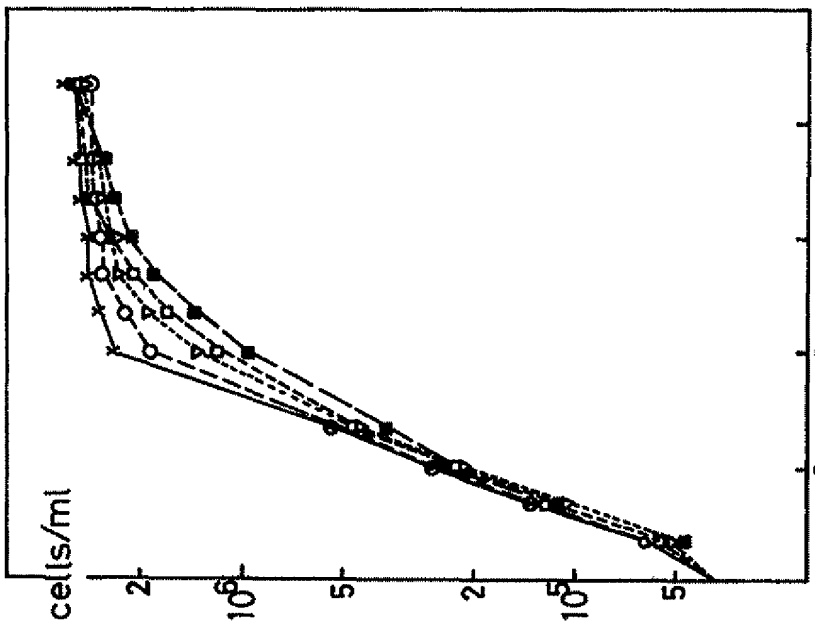

Fig. 3. Growth curves of cultures of *Phaeodactylum tricornutum* at various concentrations of zinc in the 3/4 f/10 medium: ×, control; ○, 500 μg Zn/l; ▽, 1000 μg Zn/l; □, 2000 μg Zn/l; ■, 4000 μg Zn/l.

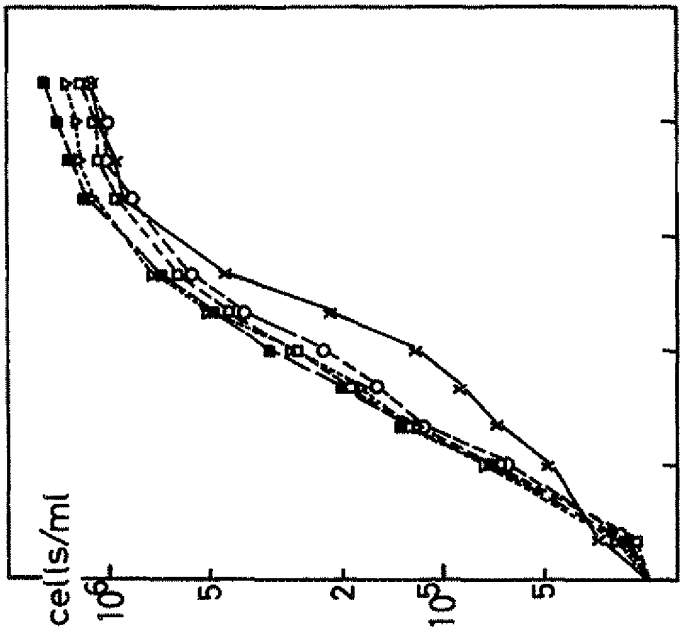

Fig. 4. Growth curves of cultures of *Phaeodactylum tricornutum* at various concentrations of Zn and Cu in the 3/4 f/10: ×, 250 μg Cu/l; ○, 250 μg Cu/l+500 μg Zn/l; ▽, 250 μg Cu/l+1000 μg Zn/l; □, 250 μg Cu/l+2000 μg Zn/l, ■, 250 μg Cu/l+4000 μg Zn/l.

TABLE VI

Growth rate of *Phaeodactylum tricornutum* (divisions/day) at various concentrations of Zn and Cu in the medium 3/4 f/10.

Zn added, μg/l →	Cu added, μg/l → 0		250	
		aver.		aver.
0	1.10		0.45	
	1.10	1.10		0.44
	1.10		0.42	
	1.10			
500	1.0		0.64	
		0.99		0.64
	0.97		0.63	
1000	1.0		0.69	
	0.95	0.94		0.69
	0.92			
	0.90		0.69	
2000	1.0			
	0.90		0.69	
	0.88	0.92		0.68
	0.88		0.67	
4000	0.87	0.84	0.73	0.71
	0.80			
			0.69	

combination with zinc is shown in Fig. 4. There was a clear toxic effect of copper (250 μg/l) which was partly overcome towards the end of the experiment. The growth retarding effect of copper was alleviated by the addition of zinc to the medium. Since there was no room for a control run in the series it is impossible to decide whether the toxic effect of the copper ions were completely relieved by the zinc ions. In two parallell series of experiments based on the 3/4 f/10–EDTA medium (Figs 5 and 6) the same tendencies were observed, *i.e.*, zinc addition to the medium lowered the growth rate somewhat, and copper alone gave a pronounced reduction in the division rate. Furthermore, the toxic effect of copper was significantly reduced upon addition of zinc. For this medium there was also a considerable difference between the highest division rate in the zinc series (Fig. 5) compared with those in the copper–zinc experiments (Fig. 6), which is taken to mean that the toxic effect of the copper (250 μg/l) was not completely removed by the addition of zinc.

Fig. 5 shows another puzzling observation, namely that addition of 2000 μg Zn/l improved the growth of the alga in this special medium (without EDTA and trace minerals). The influence of the magnesium concentration on the toxicity of zinc to *P. tricornutum* is shown in Fig. 7, which gives the cell densities at 5, 6. 7, and 9 days after inoculation in cultures containing 10,000 μg zinc/l as a function of the magnesium

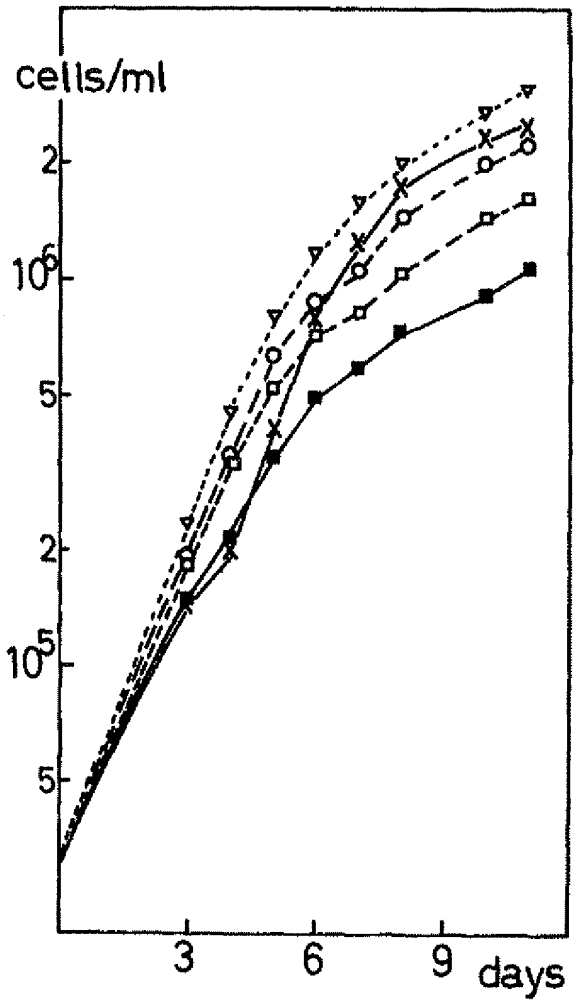

Fig. 5. Growth curves of cultures of *Phaeodactylum tricornutum* at various concentrations of Zn in the 3/4 f/10–EDTA medium: ×, control; ○, 1000 μg Zn/l; ▽, 2000 μg Zn/l; □, 4000 μg Zn/l; ■, 8000 μg Zn/l.

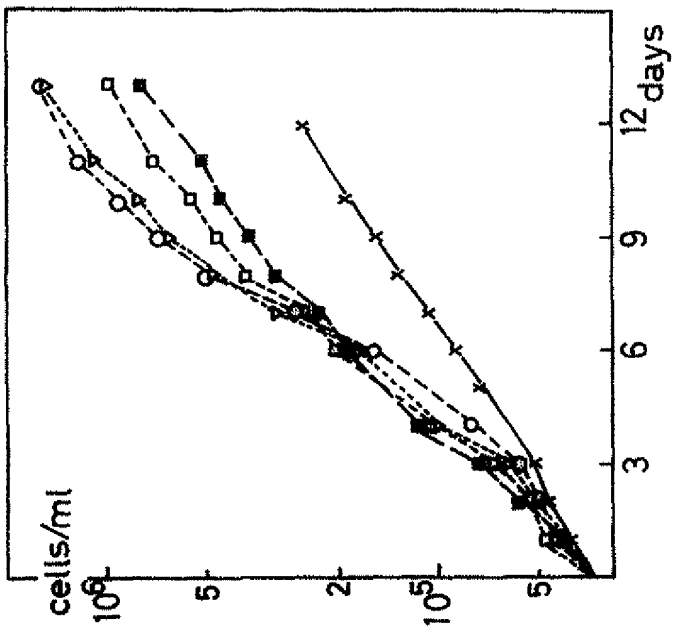

Fig. 6. Growth curves of cultures of *Phaeodactylum tricornutum* at various concentrations of Zn and Cu in the 3/4 f/10–EDTA medium: ×, 250 μg Cu/l; ○, 250 μg Cu/l + 1000 μg Zn/l; ▽, 250 μg Cu/l + 2000 μg Zn/l; □, 250 μg Cu/l + 4000 Zn/l; ■, 250 μg Cu/l + 8000 μg Zn/l.

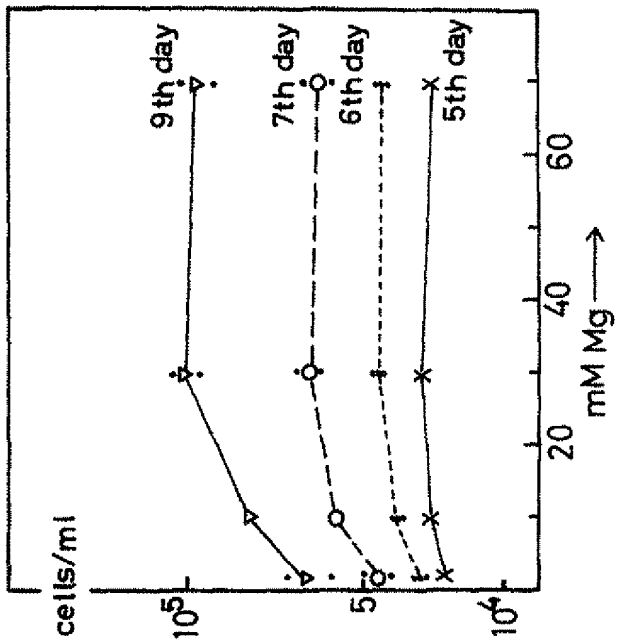

Fig. 7. Cell densities of cultures of *Phaeodactylum tricornutum* containing 10,000 μg Zn/l as a function of the magnesium ion concentration of the L/10 medium.

content. Below 30 mM magnesium in the medium, the toxic effect of the zinc ions increased with decreasing magnesium content. Reducing the magnesium concentration of the medium from 80 to 2.5 μM in the absence of excess zinc had no influence on the growth rate of the alga as seen from Fig. 8. These experiments were carried out in the modified artificial sea water medium of Lewin & Lewin (1967) in order to vary the magnesium content systematically. Weiss, Blackwelder & Wilbur (1976) have demonstrated that the growth rate of the coccolithophorid *Cricosphaera carterae* was constant over a range of $4.2 \cdot 10^{-2}$ to $1.3 \cdot 10^{-4}$ M magnesium.

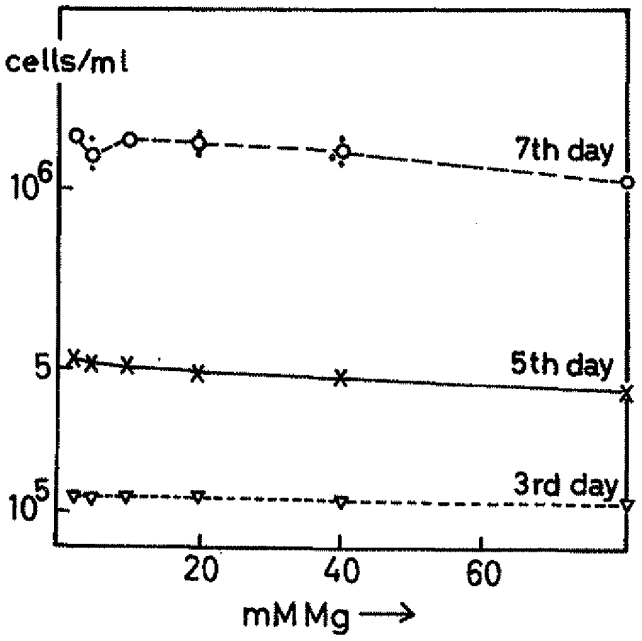

Fig. 8. Cell densities of cultures of *Phaeodactylum tricornutum* (no zinc added) as a funttion of the magnesium ion concentration of the L/10 medium.

Discussion

The present work is part of an attempt to establish the carrying capacity of fjord water for heavy metal contamination; the tolerance of three algal species to zinc and copper ions applied separately to fjord water, was studied first (Jensen, Rystad & Melsom, 1974, 1976). Due to the lack of information on the effect of heavy metals jointly applied to marine phytoplankton, it was found necessary to carry out some model experiments in batch cultures in the laboratory prior to more relevant dialysis type experiments in running fjord water.

Bliss (1939) had defined three different types of joint action of toxicants, namely: i) independent joint action; ii) similar joint action; and iii) synergistic action. The two

former may both be predicted directly from the known dosage-response curve of the constituents applied alone, while in the latter case the effectiveness of the mixture cannot be assessed from that of the individual components. One constituent synergises or antagonises the other. All three types of joint action have been detected in experiments with freshwater algae. Bartlett, Rabe & Funk (1974) claimed that combinations of copper, zinc and cadmium were similar in toxicity to equal concentrations of zinc when applied to cultures of *Selenastrum capricornutum*. Hutchinson (1974) found that copper and nickel acted synergistically and that selenium and cadmium showed antagonism to four other green algae in freshwater.

The results obtained in the present study show that joint effects of copper and zinc on marine phytoplankton could not be predicted on the basis of the toxicity of the individual metals. The dinoflagellate *A. carteri* and the diatom *T. pseudonana* gave clear cases of synergism (Figs 1, 2, Tables I, II), while the same metals acted as antagonists towards the diatom *P. tricornutum* (Figs 4, 6). For the two clones of *S. costatum* tested there can be little doubt that the effect of combinations of the metals was greater than the sum of the effects caused by the components applied separately. The sensitivity of *S. costatum* to copper did, however, limit the range of the experiments which could be carried out, and the results obtained were not so clear-cut as those on the other algae. It should be pointed out that since heavy metals may influence the length of the lag phase as well as the growth rate of the logarithmic phase and the final cell density, growth curves give more information on the effect of the metal, than that contained in a table of growth rates.

As seen from Figs 1–6 addition of copper and zinc separately or in combination did not result in clearly extended lag phases in the present experiments. Several authors have found that heavy metals cause prolongation of the lag phase more or less in proportion to the dosage, followed by normal growth (Steemann Nielsen, Wium-Andersen, 1970; Bartlett, Rabe & Funk, 1974; Zingmark & Miller, 1975). The common explanation for this phenomenon seems to be that the medium is modified during the first part of the experiment, either by exudation from living cells or by leaching from dead cells to render the heavy metal less toxic by some sort of chelation, and that the residual cells then can grow normally for the rest of the experiment. The lack of this undesirable phenomenon during the present study is satisfying. Reduced growth rates and lowered final cell yields were the main results of the treatment with heavy metals in the present work.

Three of the algae investigated (*A. carteri*, *T. pseudonana* and the Skel-0 clone of *S. costatum*) were quite similar in their tolerance to zinc alone, while clone Skel-5 of *S. costatum* was most sensitive and *P. tricornutum* extremely resistant to zinc additions. The latter species also tolerated 250 μg copper/l quite well, a concentration which caused rapid death of the other algae tested. These observations confirm those previously obtained in non-enriched sea water (Jensen, Rystad & Melsom, 1974, 1976). Quantitatively the non-enriched sea water of the fjord seems to have a slightly lower carrying capacity for zinc contamination than the 3/4 f/10 medium of the

present investigation. The latter had a content of 2.3 μM EDTA, which can chelate 74.3 μg copper/l or 76.4 μg zinc/l, and this may explain the difference observed.

Because of difficulties in keeping conditions identical, especially light and temperature, no more than 10 cultures could be grown simultaneously, so that we had to omit metal-free controls from the experiments involving both metals in the case of *P. tricornutum*. In these experiments the control cultures of the single metal treatment may be useful. It seems (Figs 3, 4) that addition of 4000 μg Zn/l to cultures of *P. tricornutum* containing 250 μg Cu/l did not completely restore growth to normal, since the control group of the zinc experiment (Fig. 3) had a growth rate of 1.1 divisions/day against 0.7 divisions/day in the combined treatment. This refers to experiments based on the medium with EDTA. Similar and even larger differences are seen upon comparison of the growth curves obtained in the 3/4 f/10–EDTA medium (Figs 5, 6). Here the highest growth rate of the combined treatment was 0.5 divisions/day against 0.9 divisions/day in the metal-free control group. Despite a clear antagonistic effect of zinc towards copper in *P. tricornutum* cultures, the toxic effect of the latter was not eliminated by addition of the former. The antagonism may be caused by a competition of the two metals for a common uptake site. In higher plants the mechanism of copper uptake seem to be different from that of zinc uptake (Antonovics, Bradshaw & Turner, 1971), and the majority of the algae investigated during the present study showed no antagonism for the copper–zinc pair. *P. tricornutum* seems, therefore, to be exceptional both in this respect and because of its extreme tolerance to zinc and copper applied separately.

Since the results indicate a common site for copper and zinc uptake in *P. tricornutum* it was of interest to investigate the influence of magnesium on the effect of zinc on this alga. The observation that the toxicity of zinc increased significantly upon reduction of the magnesium content of the medium indicates that also magnesium competed for the same site on the alga, and it may therefore be that all divalent cations act on the same site in *P. tricornutum*.

The data obtained in the present study on algal cultures indicate very strongly that complex effects on growth and development of the phytoplankton may be expected when mixtures of heavy metals are introduced into the marine habitat. Predictions of joint effects cannot be based on results obtained with the metals applied separately.

Acknowledgement

Two of the authors (G.S.B. and Å.M.) gratefully acknowledge research fellowships given by the Norwegian Research Council for Science and the Humanities. Further financial support by this Research Council is also acknowledged.

References

ANTONOVICS, J., A. D. BRADSHAW & R. G. TURNER, 1971. Heavy metal tolerance in plants. *Adv. ecol. Res.*, Vol. 7, pp. 1–85.

BARTLETT, L., F. W. RABE & W. H. FUNK, 1974. Effects of copper, zinc and cadmium on *Selenastrum capricornutum*. *Water Res.*, Vol. 8, pp. 179–185.

BLISS, C. I., 1939. The toxicity of poisons applied jointly. *Ann. appl. Biol.*, Vol. 26, pp. 585–615.

ERICKSON, S. J., N. LACKIE & T. E. MALONEY, 1970. A screening technique for estimating copper toxicity to estuarine phytoplankton. *J. Wat. Pollut. Control Fed.*, Vol. 42, pp. 270–278.

GUILLARD, R. R. L. & J. H. RYTHER, 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.*, Vol. 8, pp. 229–239.

HUTCHINSON, T. C., 1974. Comparative studies of the toxicity of heavy metals to phytoplankton and their synergistic interactions. *Wat. Pollut. Res. Can.*, Proc. 8th Can. Symp. pp. 68–75.

JENSEN, A., B. RYSTAD & S. MELSOM, 1974. Heavy metal tolerance of marine phytoplankton. I. The tolerance of three algal species to zinc in coastal sea water. *J. exp. mar. Biol. Ecol.*, Vol. 15, 145–157.

JENSEN, A., B. RYSTAD & S. MELSOM, 1976. Heavy metal tolerance of marine phytoplankton. II. The copper tolerance of three species in dialysis and batch bioassays. *J. exp. mar. Biol. Ecol.*, Vol. 22, pp. 249–256.

LEWIN, J. & R. A. LEWIN, 1967. Culture and nutrition of some apochlorotic diatoms of the genus Nitzschia. *J. gen. Microbiol.*, Vol. 46, pp. 361–367.

STEEMANN NIELSEN, E. & S. WIUM-ANDERSEN, 1970. Copper ions as poison in the sea and in freshwater. *Mar. Biol.*, Vol. 6, pp. 93–97.

WEISS, R. E., P. L. BLACKWELDER & K. M. WILBUR, 1976. Effects of calcium, strontium and magnesium on the coccolithophorid *Cricosphaera (Hymenomonas) carterae*. II. Cell division. *Mar. Biol.*, Vol. 34, pp. 17–22.

ZINGMARK, R. G. & T. G. MILLER, 1975. The effects of mercury on photosynthesis and growth of estuarine and oceanic phytoplankton. In, *Physiological ecology of estuarine organisms*, edited by F. J. Vernberg, University of South Carolina Press, Columbia, S.C., pp. 45–57.